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From the Institute for Microbiology and Molecular Biology
of the Justus Liebig University Gießen

Generation of an RNA-Polymerase I-Vector System for the Selective Mutagenesis of Influenza A Viruses

Inaugural Dissertation for Obtaining the Doctoral Degree
of the School of Natural Sciences of the Justus Liebig University Gießen

submitted by
Erich Hoffmann
from Nusbaum/Eifel

Gießen 1997

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1 INTRODUCTION

Influenza viruses belong to the family Orthomyxoviridae[1]. The representatives of this family are enveloped viruses with a single-strand RNA genome of negative polarity. In contrast to the Paramyxoviridae, the genome does not consist of one RNA molecule, but includes multiple segments.

In humans and animals, influenza viruses can cause, in addition to mild symptoms, severe febrile diseases of the respiratory tract and of the entire organism. Influenza or flu occurs periodically as a pandemic, usually originating in Southeast Asia and China. Thus, in the past century, there were five large outbreaks of influenza, in the years 1890, 1900, 1918/19, 1957, and 1968. The most severe pandemic to date, the so-called "Spanish flu" in the years 1918/1919, claimed roughly 2 million lives. Not until 1933 were Smith, Andrews, and Laidlaw able to isolate the human pathogenic influenza virus as the pathogen. The first virus of the influenza group, the fowl plaque virus (FPV), had, however, already been identified in 1900 by Centanni and Savunozzi as the pathogen of classic avian plague. Schäfer demonstrated in 1955 that this avian virus, although it causes a completely different clinical picture from the flu infection, has an antigenic relationship with the human pathogenic influenza viruses. The sequence data currently available also document the fact that there are no fundamental differences between the human, mammalian, and bird influenza viruses.

Based on type-specific determinants of the nucleocapsid and of the matrix protein, influenza viruses are categorized into three genera: A, B, and C. Based on the different antigenic properties of the viral glycoproteins hemagglutinin (HA) and neuraminidase (NA), influenza A viruses can then be further subdivided (Webster *et al.*, 1982). Nine neuraminidase variants (N1, N2,...) and fifteen hemagglutinin subtypes (H1, H2,...) are differentiated (Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1990; Röhm *et al.*, 1996). Whereas in the waterfowl viruses all

[1] derived from ορθος (gr.): right, correct and μυχα (gr.): phlegm

HA-subtypes appear (Chambers *et al.*, 1989), in the human strains only six of these subtypes occur (Laver, 1973; Webster *et al.*, 1982).

The cause for the occurrence of pandemics is the periodic change in the antigenic characteristics of a new influenza A virus subtype. As a result of double infection of a cell by two different virus strains, a new virus strain is produced by a recombination event. These new combinations occur primarily in the pharyngeal mucosal cells of swine, which can be infected by both human and avian subtypes (Scholtissek & Naylor, 1988; Kida *et al.*, 1988). In addition to the gene for the hemagglutinin, other genes can also be exchanged as whole RNA segments. From this process, referred to as antigen shift, there results a virus subtype of the human viruses that has no serologic relationship whatsoever with the variants previously circulated.

The simple flu epidemics, which occur more frequently than pandemics (every 1 to 3 years), are caused by an accumulation of gradual changes of the antigenic determinants of the viral glycoproteins (antigen drift). Virus variants developing in this manner can partially eliminate the immune response of the host and are possibly capable of continuing an infection chain despite existing basic immunity.

Uniform nomenclature standards have been introduced for designating strains of influenza isolates (WHO Memorandum, 1980). According to them, first the type, in animal isolates the host organism, the geographic origin, the number of the strain isolated, and the year of the isolation are indicated. The antigen combination of HA and NA is indicated in parentheses (e.g., A/PR/8/34 (H1N1)).

1.1 The Viral Genetic Products of the Influenza A Viruses

The genome of influenza A and B viruses consists of eight RNA segments of negative polarity (Scholtissek & Bowles, 1975; Palese, 1977). The RNA molecules can be segregated according to size on polyacrylamide gels (Scholtissek *et al.*, 1976; Ritchey *et al.*, 1977). For the association of the RNA segments with the viral proteins, the following nomenclature has been introduced: Segment 1 encodes for PB2, segment 2 for PB1, segment 3 for PA, segment 4 for HA, segment 5 for NP, and segment 6 for NA. The mRNA molecules transcribed by segments 7 and 8 are partially spliced such that all the resultant mRNAs here are translated into two proteins in each case: Segment 7 encodes for M_1 and M_2 and segment 8 for NS_1 and NS_2.

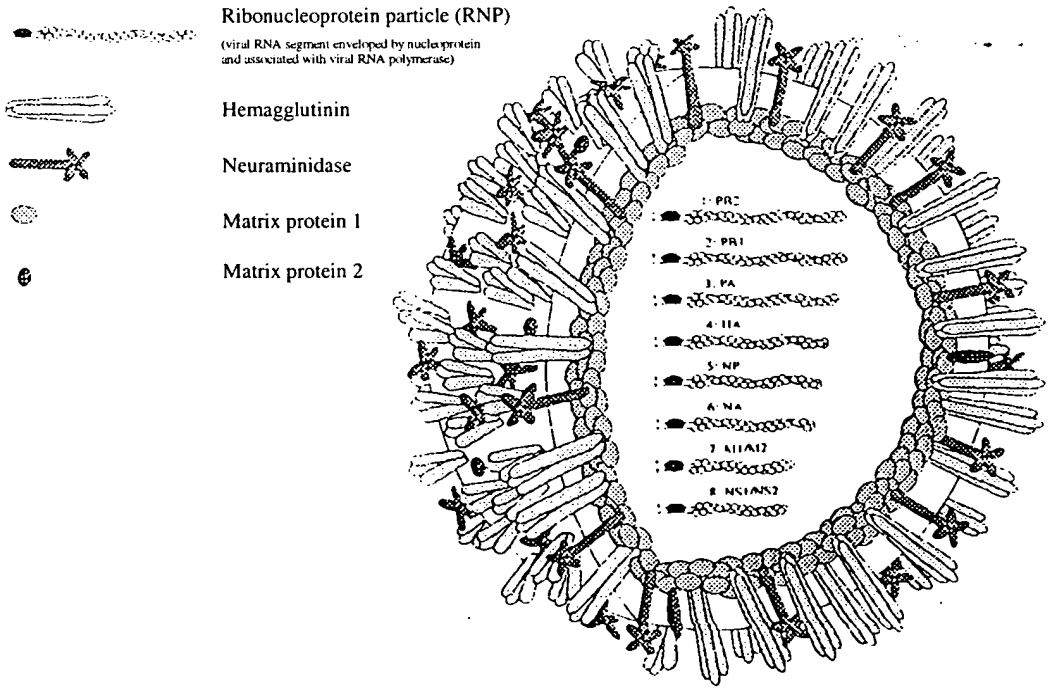


Fig. 1-1: Structure of an influenza A virus particle
The outer envelope consists of a lipid membrane, which originates in the host cell. The integral membrane proteins hemagglutinin (HA) and neuraminidase (NA) as well as the pore-forming matrix protein 2 (M_2) are embedded in the lipid bilayer. Beneath the membrane envelope, the matrix protein 1 (M_1) forms an electronmicroscopically impermeable layer, which surrounds the nucleocapsid. The nucleocapsid is composed of eight different RNA molecules of negative polarity associated with nucleoproteins to form ribonucleoprotein particles. Each genomic RNA segment is associated with numerous nucleoproteins and with a polymerase complex, consisting of the subunits PB2, PB1, and PA.

The following table summarizes some characteristics of the eight influenza segments using the example of the strain A/PR/8/34 (H1N1) (according to Fields, 1996):

Segment	Number of Nucleotides	Encoded protein	Number of amino acids	Number of molecules per virion
1	2341	PB2	759	30-60
2	2341	PB1	757	30-60
3	2233	PA	716	30-60
4	1778	HA	566	500
5	1565	NP	498	1000
6	1413	NA	454	100
7	1027	M_1	252	3000
		M_2	97	20-60
8	868	NS_1	230	-
		NS_2	121	(130-200?)

1.1.1 Hemagglutinin

The genetic product encoded by segment 4 is called hemagglutinin (HA) because of its capability of agglutinating erythrocytes. Multiple HA molecules of a virus bind to sialic acid-containing glycoprotein receptors at the beginning of an infection cycle and thus enable the attachment of a virus particle to the host cell. A prerequisite for the internalization of the virus is the cleavage of the HA_0 precursor molecules into the HA_1 and HA_2 subunits, still connected by two disulfide bridges (Klenk *et al.*, 1975; Segal *et al.*, 1992). After endocytosis of the enveloped virus particle, a pH-dependent conformation change of the trimeric HA molecule results in an unfolding of the aminoterminal end of the HA_2 subunit in the phagolysosomes (Stegmann *et al.*, 1990; Bullough *et al.*, 1994). This hydrophobic protein zone referred to as a fusion peptide initiates the fusion process between the endosomal and the viral phospholipid membrane. This releases the viral nucleocapsid into the cytoplasm, while the virus envelope with the glycoproteins remains behind in the endosomal membrane (Düzgünes *et al.*, 1992; Godley *et al.*, 1992).

The HA polypeptide newly formed during the infection cycle is channeled for post-translational modification into the rough endoplasmic reticulum. There, acylation (Veit *et al.*, 1991b), glycosylation (Elder *et al.*, 1979) as well as the correct folding and trimerization of the HA monomers (Braakman *et al.*, 1991) occurs. Then, the modified multimeric protein is transported via the Golgi apparatus to the cellular lipid membrane (Copeland *et al.*, 1986; Gething *et al.*, 1986; Garten *et al.*, 1992), where it contributes to the maturation of the newly generated virus envelope (Wiley *et al.*, 1984).

The cleavage of the HA precursor (HA_0) into two subunits HA_1 and HA_2 is critical for the infectiousness and pathogenicity of the virus (Lazarowitz & Choppin, 1975; Rott *et al.*, 1979). The cleavability and thus the pathogenicity are dependent on the number of basic amino acids at the cleavage point and on oligosaccharide side chains, which can mask the cleavage point. Two groups of proteases are considered as responsible for the cleavage: The

first group includes proteases that require only one arginine in the HA sequence for hydrolysis, e.g., plasmin, proteases similar to blood clotting factor X (Garten *et al.*, 1989; Klenk & Garten, 1994). The second group is made up of proteases that require multiple basic amino acids in the HA sequence for proteolysis. These include the subtilisin-related proteases furin and PC6 (Stieneke-Gröber *et al.*, 1992; Vey *et al.*, 1992).

1.1.2 Neuraminidase

Neuraminidase (NA) is, as the second of the two integral membrane glycoproteins, the genetic product of segment 6. The protein forms, on the virus surface, a tetramer of four identical subunits that are connected with each other by disulfide bridges (Hogue & Nayak, 1992). The NA molecule is, in contrast with the HA, anchored in the lipid membrane of the virus by a hydrophobic region on the aminoterminus. The x-ray structure shows that the tetrameric protein forms a mushroom-like structure that consists of a proximal stem region and a distal umbrella-like portion (Vargese *et al.*, 1983).

The primary function of this glycoprotein consists in separating the sialic acid from glycoproteins. The hydrolytic cleavage thus destroys the hemagglutinin receptors on the cell surface, thus helps in the release of the virus progeny and prevents the attachment of newly synthesized virus particles on already infected cells (Burnet & Stone, 1947). In addition, by the separation of sialic acids on the carbohydrate side chains of HA and NA, the initially existing self-aggregation of virus particles is slowly broken down and thus infectious individual virus particles are not released until then (Palese *et al.*, 1974).

The removal of sialic acid residues from the hemagglutinin molecules results in improved cleavage and, thus, activation of the HA molecule. The glycosidase activity enables it, after infection, to pass through the mucin-rich layer in the respiratory tract of the host organism and thus to reach the epithelial cells of the lungs (Fields, 1996).

1.1.3 Matrix Proteins

The membrane (matrix) proteins M_1 and M_2 are encoded by segment 7. M_1 is encoded by a collinear transcript; M_2, in contrast, by a spliced mRNA, whereby in the second exon the open reading frame of the M_2-encoding sequence overlaps partially with that of the M_1 encoding sequence in a second reading frame (Allen *et al.*, 1980; Lamb & Chopin, 1981; Lamb & Lai, 1981). The M_1 protein represents the largest protein component by quantity in the virion and forms an approximately 6-nm-thick, closed protein envelope under the lipid membrane (Schulze, 1970; Skehel & Schild, 1971). Because of its position, interaction with cytoplasmatic components of the integral membrane glycoproteins is very likely (Choppin *et al.*, 1972; Bucher *et al.*, 1989). The interaction with the cytoplasmatic components of the glycoproteins as well as the interaction with the RNP particles indicates a critical role in the synthesis of the virus particles.

The M_2 protein is present only in small numbers in the virus particle (Zebedee & Lamb, 1988; Helenius *et al.*, 1992). The palmitoylated homotetramer consists of two dimers linked by disulfide bridges. This multimer represents a membrane-anchored protein that forms an ion channel (Veit *et al.*, 1991a; Pinto *et al.*, 1992). This ion channel, which can be blocked by amantadine, can regulate the pH value in the *trans* section of the Golgi apparatus and thus indirectly alter the pH-dependent conformation of the HA molecule (Sugrue & Hay, 1991; Skehel, 1992; Ciampor *et al.*, 1992).

During the early stages of the infection, a drop in the pH value in the phagolysosomes results in the conformational modification of HA and in the fusion of the viral and cellular lipid membranes. The low pH value inside the virus particle also results in a break in the contact between the M_1-layer and the RNP complexes (Armstrong & Dimmock, 1992; Zhirnov, 1990). The two processes result in the release of RNP particles into the cytoplasm, which, after transport into the cell nucleus, trigger the replication cycle of the virus.

1.1.4 Nonstructural Proteins

Segment 8 carries the genetic information for two proteins (Inglis *et al.*, 1979). Since, for a long time, these could only be detected in infected cells and not in virus particles, they were called "nonstructural proteins" NS_1 and NS_2. The NS_1 protein is translated by an unspliced mRNA. Through splicing, a truncated mRNA develops that is used for the translation of the NS_2 protein. The two proteins have identical amino acids on the aminoterminal end; the reading frame changes from the spliced point, yielding a different protein sequence with NS_2 (Alonso-Caplen *et al.*, 1991).

The NS_1 protein is a phosphoprotein which is detectable in polysomes, in the nucleus and nucleolus (Privalsky *et al.*, 1981; Krug *et al.*, 1975). The nuclear transport signal consists of two sequence sections (Greenspan *et al.*, 1988). Two primary functions have been described for the NS_1 protein. Accordingly, on the one hand, NS_1 regulates the export of mRNA from the nucleus; on the other, NS_1 inhibits the splicing of pre-mRNAs. The NS_1 protein inhibits the export of spliced viral and cellular mRNA (Fortes *et al.*, 1994; Qian *et al.*, 1994; Wolf *et al.*, 1996), because it specifically binds to poly A sequences and thus inhibits the export of any poly A-containing mRNA (Qiu *et al.*, 1994).

In contrast to NS_1, it was possible to detect small quantities of NS_2 in virions (Richardson & Akkina, 1991). Here, it seems to be present in association with the M_1 protein (Yasuda *et al.*, 1993). In the cell, it could be detected both in the cell nucleus and in the cytoplasm (Smith *et al.*, 1987; Greenspan *et al.*, 1985). NS_2 serves as an adapter molecule between the viral RNP complexes and the nuclear export machinery (Palese *et al.*, 1997). Consequently, it is also referred to as "nuclear export protein" (NEP).

1.1.5 Nucleoprotein

The RNA segment 5 encodes for the basic nucleoprotein (NP). The analysis of the nucleotide sequences and the derived amino acids sequences results in the subdivision of two classes of the NP-protein: an avian and a human type (Buckler-White & Murphy, 1986). Scholtissek's

research group showed that the nucleoprotein represents a significant factor for the species-specificity of the influenza A virus strain (Scholtissek *et al.*, 1985). Thus, the human virus strain A/HK/1/68 cannot complement the temperature-sensitive phenotype of the NP-protein in the avian virus strain A/FPV/Rostock/1/34 in chicken embryo fibroblast (CEF) cells. In contrast, reassortants are formed after double infection of this strain in mammalian cells (MDCK cells). The daughter viruses produced also replicate in MDBK cells, but not in CEF cells (Scholtissek *et al.*, 1978).

After the synthesis of the NP-protein in the cytoplasm, the NP molecules accumulate in the cell nucleus. For nuclear transport, a signal sequence that is necessary and sufficient for the nuclear localization has been described (Davey *et al.*, 1985). The karyophilic sequence characterized is differentiated from the basic amino acid residues found in other nuclear proteins. More recent investigation shows that even NP mutants which do not have this sequence are localized in the cell nucleus. The sequence important for the import function has been found on the N-terminal end of the protein (Wang *et al.*, 1997; Neumann *et al.*, 1997). The NP-protein is phosphorylated, whereby during the course of an infection a change in the phosphorylation pattern has been noted (Kistner *et al.*, 1989). The nucleoprotein is a primary structural component of the ribonucleoprotein particle and binds cooperatively both to the cRNA and to the vRNA. Stoichiometric calculations show that roughly 20 nucleotides are covered by one NP molecule. The viral mRNA, in contrast, is not enveloped by NP protein (Hay *et al.*, 1977b).

Using the "two hybrid system" in yeast, a cellular protein that interacts in the cytoplasm with the nucleoprotein has been identified (O'Neill & Palese, 1995) and is called NP-I. In parallel studies, it has been described as part of the general machinery for the transport of proteins into the cell nucleus, beginning with the interaction of this protein factor (karyopherin α) on the nuclear transport signal sequence (Radu *et al.*, 1995).

1.1.6 Polymerase Proteins (PB1, PB2, PA)

The viral RNA-dependent RNA-polymerase is encoded by the three largest segments (Kaptein & Nayak, 1982). The three proteins form a heterotrimeric polymerase complex which is composed of the two basic subunits PB1 and PB2 as well as the acidic component PA (Murti *et al.*, 1980; Bram *et al.*, 1983; St. Angelo *et al.*, 1987; Krug *et al.*, 1989). This complex is detectable in the nucleus of infected cells (Jones *et al.*, 1986). For transport, specific nucleus localization signals are necessary (Jones *et al.*, 1986; Mukaigawa & Nayak, 1991). The RNA polymerase complex has a large number of enzymatic activities and thus assumes a central role in the replicative cycle of the influenza viruses. Thus, with the help of the polymerases, the viral vRNAs, cRNAs, and mRNAs are synthesized. In a primer-independent reaction, the vRNA with negative polarity is replicated to the complementary plus-strand cRNA (Young & Content, 1971; Hay *et al.*, 1982). The cRNA is then used as a template for the vRNA synthesis. As the first in the influenza infection, the parental vRNA is, however, transcribed into mRNA molecules in a primer-dependent synthesis (Hay *et al.*, 1977b; Plotch *et al.*, 1981). The necessary starter fragments are obtained by separation of 9-15 nucleotides from cellular mRNAs (Caton & Robertson, 1980). The recognition of the 5' cap structure of the cellular mRNAs is mediated by the subunit PB2 such that after endonucleolytic cleavage (preferably after a purine residue) the cellular oligonucleotide hybridizes on the 3' end of the vRNA and a 3' hydroxyl residue is available for the start of the viral mRNA transcription (Ulmanen *et al.*, 1981, 1983; Blaas *et al.*, 1982a, 1982b; Seong & Brownlee, 1992a; Hagen *et al.*, 1994). The transcripts created then have on their 5' end heterologous host cell sequences including the 5' cap structure (Skehel & Hay, 1978; Krug *et al.*, 1979; Desselberger *et al.*, 1980; Dhar *et al.*, 1980; Beaton & Krug, 1981). The synthesis of a poly A segment on the 3' end of the viral mRNAs is also catalyzed by the viral polymerase complex (Hay *et al.*, 1977a, 1977b; Robertson *et al.*, 1981; Luo *et al.*, 1991).

1.2 Replication and Transcription of the Influenza Genome

Influenza virus particles bind via their HA surface protein to the N-acetyl neuraminic acid or the 9-O-acetyl neuraminic acid on the cell surface. The cytoplasm membrane then endocytotically surrounds the bound virus particles and internalizes them in vesicles in the cell. Through the drop in the pH value in the endosomal vesicles after fusion with the lysosomes, the HA protein changes its conformation. Thus, the hydrophobic sequence of amino acids on the N-terminus of the HA_2 subunit can unfold and become embedded in the endosomal membrane, which results in the fusion of the viral and the cellular lipid bilayers. The acidification also triggers the interaction of the NP-proteins with the M_1 proteins in the interior of the virions. The nucleoprotein complexes are thus individually released from the cytoplasmatic vesicles and then transported through the cell pores into the cell nucleus, where the replication and transcription occurs (Helenius, 1992).

The eight RNA segments of the influenza viruses have highly conserved sequences on their 5' and 3' ends (Parvin *et al.*, 1989; Yamanaka *et al.*, 1990), which are involved in the regulation of the transcription and replication as well as for packaging in the virus particles generated (Honda *et al.*, 1987; Luytjes *et al.*, 1989). The first 13 nucleotides of the 5' end and the first 12 nucleotides of the 3' end are identical for all eight segments. These two end regions have nucleotide sequences complementary to sections of each other (Robertson, 1979; Desselberger *et al.*, 1980; Stoeckle *et al.*, 1987). By hybridization of the two ends, RNA secondary structures can develop. On each of these partially double-stranded regions of the individual RNA segments the viral RNA-polymerase-complex is present bound in the virus particle (Honda *et al.*, 1987; Hsu *et al.*, 1987).

The analysis as to whether both terminal sequences are necessary for the polymerase binding has yielded sometimes different results. For a long time, binding on the 3' end of the vRNA was considered adequate for the polymerase-RNA interaction (Luytjes *et al.*, 1989; Parvin *et al.*, 1989; Luo *et al.*, 1991; Piccone *et al.*, 1993).

In vitro investigations yielded polymerase binding sites on the nucleotide positions 9 through 11 on the 3' end of the vRNA (Seong & Brownlee, 1992a, 1992b; Fodor *et al.*, 1993).

The uridine residues, usually 6, on the 5' end of the vRNAs are necessary for the polyadenylation of the mRNA (Luo *et al.*, 1991). A mechanism is assumed whereby the RNA-polymerase can synthesize a poly A sequence including roughly 200 nucleotides by repeated copying of the uridine-rich section.

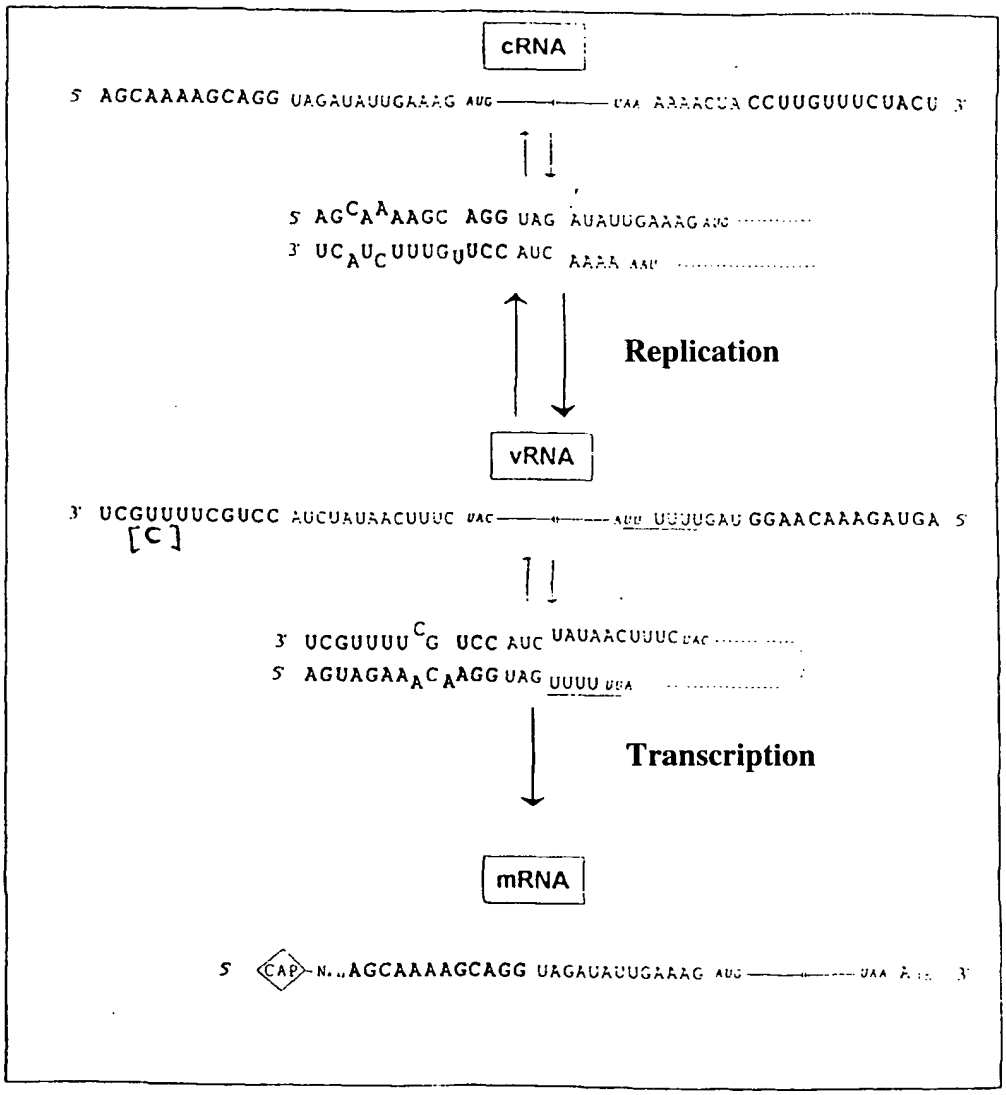


Fig. 1-2: Transcription and replication in influenza A viruses
The nucleotides depicted include the highly conserved terminal sequences (fatty) present in all eight segments as well as segment-specific nucleotides (in the example of the seventh segment). Replication occurs in a primer-independent reaction in which the genomic vRNA serves as the template for the cRNA. The mRNA, which bears a heterologous host cell sequence with 5' cap structure on its 5' end, is formed in a primer-dependent reaction. On the 3' end, the mRNA has a poly A segment for whose synthesis the 5-7 uridine residues (underlined) of the vRNA template are essential.

1.3 RNA-Polymerase I Transcription System

The ribosomal RNA (rRNA) of all eukaryons is transcribed in the nucleolus by the RNA-polymerase I. Human cells contain roughly 200 rDNA transcription units per haploid genome, which are localized on five different chromosomes. The so-called *spacer*-DNA, which is not transcribed, is located between the tandemly arranged rDNA transcription units. The ribosomal RNAs are transcribed together as a pre-rRNA precursor molecule. This 45S primary transcript is then processed through multiple intermediate steps into the final 18S, 5.8S, and 28S rRNAs.

Through the establishment of *in vitro* transcription systems, it was possible to clarify that the 5' and 3' ends of the pre-rRNA are formed precisely. In contrast to polymerase II transcripts, the polymerase I transcript has no cap structure and no poly A end. On the 5' end, the rRNA has a 5'-triphosphate group; and on the 3' end, a defined 3'-OH residue. These terminal structures thus correspond to the ends of genomic RNA molecules (vRNA) of influenza A-viruses. To enable the vRNA synthesis, the cDNA of the influenza segment 4, which encodes the glycoprotein hemagglutinin, was inserted nucleotidelike in reverse orientation between the rDNA promoter and the rDNA terminator (Zobel *et al.*, 1993). The rDNA promoter hereby includes the region from -254 through -1 of the RNA-polymerase I promoter of the mouse. The region from +571 through +745, referenced from the 3' end of the 28S rDNA of the mouse, was used as rDNA terminator (La Volpe *et al.*, 1985; Bartsch *et al.*, 1987). This terminator region includes two highly conserved 18 bp long sequence elements, which are called "Sal boxes" in reference to the recognition sequences for the restriction endonucleases *Sal*I contained therein (Grummt *et al.*, 1986).

Using ascites cell extracts, it has been demonstrated *in vitro* that specific transcripts, whose 5' ends match the first hemagglutinin nucleotide to be transcribed, are formed with the help of this transcription system (Zobel *et al.*, 1993). The termination process of the pre-rRNA transcripts (Platt, 1986) takes place in a two-step process (Kuhn & Grummt, 1989). Through

the binding of the cellular termination factor TTF-1 onto the "Sal box" (Smid *et al.*, 1992), the primary termination reaction takes place 12 bp upstream from this sequence. In a following processing reaction, additional nucleotides are removed from the primary 3' end and a specific secondary 3' end develops farther upstream from the previously formed 3' end of the primary transcript (Zobel *et al.*, 1993).

For the *in vivo* analysis of the vRNA formed by RNA-polymerase I transcription, the HA-encoding sequence was replaced nucleotidelike by the reporter gene chloramphenicol acetyltransferase (CAT). The transfection of these plasmid constructs in mouse fibroblast cells and then superinfection with influenza helper viruses resulted in the expression of the reporter gene (Neumann *et al.*, 1994).

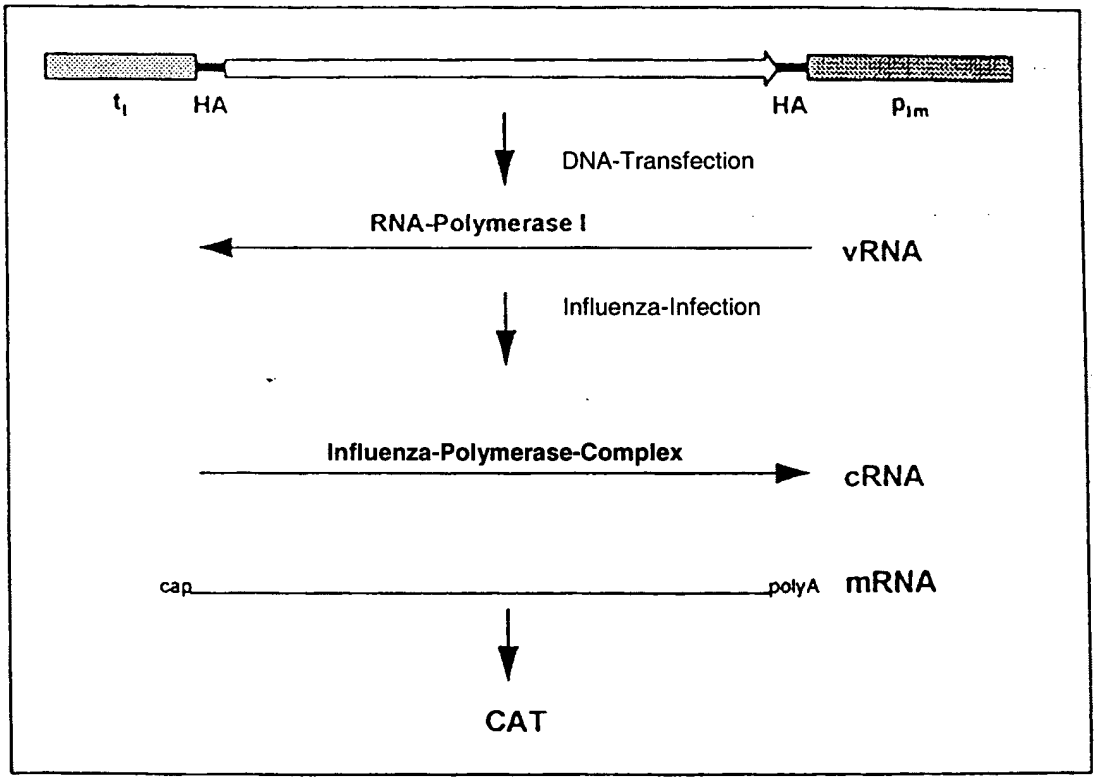


Fig. 1-3: RNA-Polymeras I Transcription Syst m
The nontranslated end regions of the HA-cDNA in negative orientation are located between the murine RNA polymerase I promoter (p_{Im}) and terminator (t_I). The HA encoding sequence has been replaced by the reading frame of the reporter gene CAT. After DNA transfection, the cellular RNA polymerase I synthesizes RNA molecules which correspond on their 5' and 3' ends nucleotidelike to viral RNA. After infection with influenza helper viruses, the viral polymerase complex recognizes the end structures, which results in the synthesis of cRNA and mRNA and, after translation, in the formation of the reporter protein CAT.

Passage experiments demonstrated that the hybrid vRNA molecules formed by the RNA-polymerase I transcription system can be amplified and also packaged in daughter virus particles (Neumann & Hobom, 1995).

Thus, two systems are available for the selective mutagenesis of influenza A viruses: The plasmid-based DNA transfection system established by Neumann *et al.* and the RNP transfection system established by Luytjes *et al.* With the latter system, recombinant DNA is first synthesized by *in vitro* synthesis under the control of the T7 promoter. Then, the RNA formed is incubated with polymerase proteins and nucleoproteins purified from virus particles for the "reconstitution" of RNP complexes. These recombinant RNP particles are finally transfected into eukaryotic cells. After infection with an influenza helper virus, the RNA inserted is amplified, expressed, and packaged. A methodological disadvantage of the RNP transfection system consists in the fact that, for the formation of the RNP complexes, an *in vitro* RNA synthesis is first performed on a preparatory scale, and, furthermore, the four viral proteins must be purified in native conformation on a large-scale from virus preparations. These labor-intensive steps are eliminated with the direct plasmid transfection using the RNA-polymerase I transcription system. The synthesis of the vRNA in the eukaryotic cells has, in contrast to a prior *in vitro* transcription, the advantage that the test results can be obtained independent of any RNA degradation processes which may occur.

1.4 Research Objectives

Within the framework of this research, through selective mutagenesis of the end regions of the influenza A-RNA segments, the significance of the nontranslated regions is to be investigated. For this, specific mutations are introduced into the 5' and 3' end regions of the viral RNA segments with the RNA polymerase I transcription system, followed by verification of the resultant effects by means of CAT analysis. These analyses will contribute to obtaining information concerning the significance of these sequence regions in the molecular processes of replication, transcription, and packaging of the viral RNA molecules.

The more accurate knowledge of the sequence elements that are essential in the viral lifecycle further enables a methodological improvement through the selective design of cloning vectors. A vector system with a multiple cloning position has the advantage that a large number of constructs can be generated for homologous and heterologous RNA and protein expression.

A further improvement of the RNA polymerase I system can be obtained by the insertion of a readily detectable reporter gene. Consequently, it is to be investigated whether the green fluorescing protein is detectable in the influenza A mediated protein expression. This fluorescence marking of live and, for example, infected or DNA transfection cells offers good technical optimization possibilities in the cotransfection and co-expression of recombinant genes in the influenza system, through immediate detection of this protein expression. These investigations should serve to provide foundations for the production of recombinant influenza viruses and live vaccines.

2 MATERIALS AND METHODS

2.1 Cloning and Analysis of DNA

2.1.1 Bacterial Strains

The bacterial strains used in this research are derivatives the *Escherichia coli* strain K12. Based on their genetic properties, many bacterial strains are particularly well suited for various special tasks.

For general clonings, the strains HB101, XL1, and SURE were used. However, after preparation, many restriction sites could not be cut from these cells. The reason for this lies in the synthesis of Dcm- or Dam-methylases. The Dam-methylase catalyzes the transfer of a methyl group of S-adnosyl methionine to the N^6 position of the adenosine residue in the sequence GATC. Thus, the methylation of the adenosine in the sequence TCTAGATC inhibits, for example, cleavage with *Xba*I. Consequently, for the amplification and preparation of plasmid DNA without these inhibiting methyl groups, the dam$^-$-strain GM33 was used. The strains BMH71/18, GC382, and BL21 were used for the inducible protein expression in *E.coli.*

2.1.2 Isolation of Plasmid DNA Using the Minilysat Method

Bacteria were mixed in LB (Luria Bertani) medium under addition of ampicillin (100 μg/ml) and harvested by centrifugation after 10 to 15 hours of growth under agitation at 37°C. The isolation of plasmid DNA is carried out according to the protocol of the alkaline isolation method (Birnboim & Doly, 1978). First, 1-1.5 ml of an overnight culture is centrifuged for 5 minutes at 6000 rpm in a tabletop centrifuge (Biofuge A, Heraeus). The sediment is resuspended in 100 μl of solution I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA; pH 8.0). By addition of 200 μl of solution II (200 mM NaOH, 1% SDS), the bacteria are

lysed and both the genomic and the plasmid DNA denatured. After 5 minutes, 150 µl of solution III (3 M sodium acetate; pH 4.8) is added. Only the plasmid DNA is renatured by this neutralization step, whereas SDS protein complexes precipitate and are separated from the plasmid DNA along with chromosomal DNA by centrifugation (10 minutes, 12,000 rpm). The plasmid-containing supernatant is precipitated with 350 µl isopropanol. The plasmid DNA sediments by centrifugation (15 minutes at 12,000 rpm) and is then washed with 80% ethanol (5 minutes at 12,000 rpm), dried, and depending on the application, mixed in from 20 to 50 µl H_2O.

2.1.3 Preparation of Plasmid DNA through Anion-Exchanger Columns

The isolation of relatively large quantities of plasmid DNA is carried out using the "Midi Kit" from the company Qiagen. An advantage of this method is the rapid, clean preparation of DNA which was used primarily for transfection experiments with tissue culture cells. After absorption of the cell lysate on a column made from a modified silica gel, the column is washed with low concentrations of NaCl, and then, the DNA is eluted with high concentrations of NaCl.

100 to 150 ml of an overnight culture are centrifuged under cooling for 5 minutes at 5000 rpm (GSA-Rotor, Sorvall RC-5b-Centrifuge). The bacteria sediment is resuspended in 4 ml chilled solution P1 (50 mM Tris/HCl, 10 mM EDTA, 100 µg/ml RNase A; pH 8.0). Then, 4 ml of solution P2 (200 mM NaOH, 1% SDS) is added. After addition of solution P3 (3.0 potassium acetate; pH 5.5), the plasmid DNA is renatured. During the subsequent centrifugation (20 minutes at 10,000 rpm; 4°C), cellular debris and genomic DNA are sedimented out. The plasma-containing supernatant is run over a QIAGEN tip 100-column previously equilibrated with 4 ml QBT buffer (0.75 M NaCl, 0.05 M MOPS, 15% ethanol, 0.15% Triton; pH 7.0). In order to completely remove RNA and protein residues, the column is washed twice with 10 ml QC-washing buffer (0.1 M NaCl, 0.005 M MOPS, 15% ethanol; pH 7.0) each time. The plasmid DNA bound to the column material is eluted by the addition of 5 ml QF-elution buffer (1.25 M NaCl, 0.05 Tris/HCl, 15% ethanol; pH 8.5) and precipitated with 3.5 ml isopropanol.

2.1.4 Precipitation of Nucleic Acids

For concentration or decontamination of nucleic acid solutions, nucleic acids may be precipitated as sodium or ammonia salt. With high concentrations of monovalent cations, DNA or RNA precipitates by addition of ethanol or isopropanol. For this, the solution is reacted with 1/10 volumes of 3 M sodium acetate or ammonium acetate and thoroughly mixed. After addition of 2 to 3 volumes of ethanol or 0.7 volumes of isopropanol, precipitation occurs for from 5 to 15 minutes. The precipitate is sedimented by centrifugation (10 to 30 minutes) at 12,000 rpm. The sediment is washed with 80% ethanol and dried. The DNA thus obtained can now be mixed with water.

2.1.5 Cleavage of DNA with Restriction Endonucleases

A group of enzymes that cleave sequence-specifically are referred to as restriction endonucleases. The restriction enzymes of type II or type IIs are particularly well suited for the analysis and modification of plasmids and PCR products. They recognize one palindromic sequence usually including 4 to 6 (8) bp, and cut inside (type II) or outside (type IIs) this recognition sequence. The cleavage produces a hydroxyl group on the 3' end of the DNA and a phosphate group on the 5' end. After cleavage, depending on the enzyme used, the DNA can have smooth, 3' overhanging, or 5' overhanging ends.

The reaction preparations are incubated in their buffer conditions according to the recommendations of the manufacturing company (Biolabs, AGS).

2.1.5.1 Preparative Cleavage

For preparative cleavage preparations, 10 to 50 μg plasmid DNA are used. For this, according to manufacturer's instructions, cleavage preparations with total volumes of from 100 to 300 μl are prepared. After an incubation period of from 3 to 10 hours, an aliquot of the preparation is checked for completeness of cleavage on an agarose gel.

2.1.5.2 Partial Cleavage

If, in addition to the desired cutting sites, a plasmid also has additional sites with an identical recognition sequence, a partial cleavage must be performed. In order to prevent all cutting sites from being completely used, a reaction kinetic is established to determine the time that delivers the desired fragment in the greatest possible quantity. In order to determine the correct point in time, during incubation at defined time intervals, an aliquot of the cleavage preparation is taken and the enzymatic cleavage is stopped by freezing in liquid nitrogen. The point in time of the desired cleavage specimen can be determined separately by gel electrophoresis. The primary preparation is then further cleaved until this point in time.

2.1.6 Modification of DNA Fragments

2.1.6.1 Klenow Reaction

In order to be able to ligate incompatible 5' overhanging ends, the overhanging ends are transformed into smooth ends by filling up the single-strand ends using Klenow polymerase.

A typical reaction preparation contains, in a total volume of 25 µl, from 1 to 2 µg DNA, 2.5 µl 10x buffer (60 mM Tris/HCl, 60 mM NaCl, 60 mM $MgCl_2$, 0.5% gelatin, 10 mM gelatin, 10 mM DTT; pH 7.5), 1 µl of each necessary dNTP (10 mM), and 2 µl of Klenow polymerase (5 units/µl; Strategene). The preparation is incubated for 15 minutes at 22°C.

2.1.6.2 T4 Polymerase

The 3' overhanging ends are degraded to form smooth ends exonucleoticly by means of T4 DNA polymerase. From 0.5 to 2 µg DNA are incubated with two units of T4 DNA polymerase in 25 µl T4 polymerase buffer (5 mM Tris-HCl, 10 mM ammonium sulfate, 0.01 mM EDTA, 1 mM 2-mercaptoethanol) at 37°C. In order to possibly reconstruct double-strand DNA degraded by the 3'-5' exonuclease activity, 4 µl each of a 2 mM solution of all

four deoxyribonucleotides was added to the preparation after 5 minutes. After 3 minutes at 37°C, the preparation was extracted with phenol chloroform and the DNA precipitated with ethanol.

2.1.6.3 Alkaline Phosphatase

Alkaline phosphatase cleaves 5' phosphate groups from RNA or DNA ends. It is possible by using this enzyme to prevent monomolecular religation in the subsequent ligation reaction after linearizing cleavage of a vector with a restriction enzyme. To enable a ligation between insertion fragment and vector, it suffices that the fragment used have one 5' phosphate group on each of the two ends. A complete covalent binding of the fragments then occurs after the transformation by kinase and DNA ligase or by repairing enzymes in the bacteria cell. An alkaline phosphatase isolated from *Pandalus borealis (shrimp alkaline phosphatase*, USB.) was used. In addition to the high enzyme activity at 37°C, the heat lability permits a slight inactivation such that subsequently an aliquot of the phosphatase preparation can be used directly for a ligation reaction.

The reaction preparation consists of 20 mM Tris/HCl, 10 mM $MgCl_2$ and the DNA used in a concentration of 20 ng/μl. Depending on the substrate, the enzyme quantity added fluctuates between 0.1 and 0.5 U. After 30 to 60 minutes incubation at 37°C, the reaction is stopped by inactivation of the alkaline phosphatase (15 minutes at 65°C).

2.1.6.4 T4 Polynucleotide Kinase

T4 polynucleotide kinase catalyzes the transfer of a γ-phosphate group of ATP to the 5' hydroxyl groups of single-strand or double-strand DNA. After amplification of DNA fragments using inverse PCR, 5' phosphate groups are necessary to enable a ligation with the 3' hydroxyl groups. From 0.5 to 2 μg DNA with 10 units of T4 polynucleotide kinase are incubated at 37°C for 60 minutes in 50 μl of reaction buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT; pH 7.6).

2.1.7 Separation of DNA Fragments by Gel Electrophoresis

2.1.7.1 Agarose Gel Electrophoresis

Agarose gels enable separation of DNA fragments of 0.3 to 20 kb. The hydrated agarose strips, which consist of D-galactose and 3,6-anhydro-L-galactose units, form a molecular network in the gel. The porosity of this network depends on the agarose concentration. To produce agarose gels, agarose (Gibco/BRL, Eggenstein) is boiled in the microwave in 1 x agarose buffer (40 mM Tris-HCl, 5 mM Na-acetate, 1 mM EDTA, pH 7.8), cast in flat bed trays and a PVC comb used. The DNA fragments to be separated are reacted with 1/10 volume blue solution (50% [v/v] glycerol; 50 mM EDTA; 0.005% [w/v] bromophenol blue, Merck, Darmstadt); and the gel pockets filled. In other instances, 10% (v/v) gel loading buffer, which can also contain two blue dyes (bromophenol blue, xylene cyanol), in addition to glycerol and EDTA, was used to weight the samples of the DNA. The blue-stained dye front enables keeping track of the electrophoresis. The separation of the fragments occurs at a constant current flow of 80 to 100 mA. After termination of the electrophoresis, the gel is placed in an ethidium bromide solution for 10 to 15 minutes to stain the DNA bands. Ethidium bromide is a fluorescing substance that intercalates in the DNA and thus enables observation of the DNA in UV light (366 or 254 nm). The bands visible in the UV range can be photographed with a CCD camera (BIOPRINT DS BIOPROFIL Video Hardware; Vilber Lourmat, Marne La Vallee, France) or an instant camera (Polaroid MP 4 Land; Polaroid Type 667 black and white Land film, Kodak orange filter No. 22) with UV transmitted light (254 nm). The fragment sizes can be determined by comparison with various length standards. The following length standards for determination of the sizes of gel electrophoretically separated DNA fragments were used: A pAT153/*Pvu*II/8/PB1-DNA cleaved with *Pst*I, *Pst*I/*Bam*HI-, *Hin*dIII together with lambda dv1-DNA cleaved with *Hae*III yields the following fragment sizes [bp]: 5664, 4133, 3136, 2528, 1713, 1310, 890, 845, 686, 534, 460, 362, 352, 272, 223, 213, 212, 178, 142, 131, 83, 40, 36, 18, 13.

2.1.7.2 Polyacrylamide Gel Electrophoresis

For the separation of DNA fragments between 30 and 300 bp, vertical, 7.5% polyacrylamide gels are used. The gel solution is made up of the following components: 1.88 ml 40% acrylamide/bisacrylamide stock solution, 1.0 ml 10x E-buffer, 6.62 ml H_2O, 0.5 ml 1% AMPS solution, 10 μl TEMED. This gel mixture is placed between two glass plates for polymerization. The gel is mounted in a vertical chamber (BIORAD, Munich) and run with running buffer (40 mM Tris-HCl, pH 8.3, 20 mM Na acetate; 2 mM EDTA) at a constant voltage of 150 volts. As a length standard, a fragment mixture of *HaeIII*-cleaved lambda dv1-DNA with the following fragment sizes [bp] was used: 1713, 1310, 890, 686, 534, 460, 362, 352, 272, 223, 213, 212, 178, 142, 131, 83, 40, 36, 18, 13.

2.1.8 Isolation of DNA Fragments

DNA fragments are cut out of an agarose or polyacrylamide gel according to their staining in the ethidium bromide bath under longwave UV light with a scalpel after electrophoretic separation. This is followed by isolation of the DNA from the gel.

2.1.8.1 "Gene-Clean" Method

The isolation of DNA fragments with a size greater than 300 bp from preparative agarose gels is performed according to a modified instruction from Vogelstein and Gillespie (1979). The DNA fragment cut out of the agarose gel is reacted depending on the mass of the gel with from 400 to 600 μl of a saturated potassium iodide or sodium iodide solution (6 M). After a 5- to 10-minute incubation at 60°C, the agarose melts and the DNA is present in solution. After addition of from 1 to 3 μl silica matrix (produced in-house by J. Hirzmann), because of the highly saline conditions, there is adhesion of the DNA to the silica particles during a 10-minute incubation at room temperature. To clean the DNA silica particle complex of KI or NaI and agarose residues, this is followed by washing three times with a low-molar washing buffer (10 mM Tris/HCl; pH 7.5, 10 mM NaCl, 1 mM EDTA in 50 to

70% ethanol). Depending on the further use of the DNA, the DNA silica particle complex is mixed in 10 to 200 μl H_2O after the washing procedure and incubated for 2 to 5 minutes at 60°C. The double-stranded DNA is released from the silica matrix with a low salt concentration and is transferred into the aqueous solution. By means of two subsequent centrifugation steps (5 minutes at 12,000 rpm), the silica particles are separated from the DNA. An aliquot of the aqueous supernatant can then be applied to an analytical agarose gel to monitor purity and quantity.

2.1.8.2 Electroelution

For isolation by electroelution, the piece of gel is transferred to a dialysis tube (Visking type 20/30, Roth, Karlsruhe). The dialysis tube, knotted on both ends, is placed in an electrophoresis chamber. By application of 100 volts, the DNA is eluted into the buffer. The buffer is removed from the dialysis tube, subjected to phenol/chloroform purification, and then precipitated.

2.1.8.3 Isolation from Polyacrylamide Gels

DNA fragments up to 300 bp can be isolated from polyacrylamide gels by diffusion. The piece of gel cut out is crushed into small pieces and coated with dilution buffer (0.1 M Tris-HCl, 0.5 M NaCl, 5 mM EDTA; pH 8.0). The DNA diffuses from the gel matrix into the dilution buffer overnight at 60°C in a water bath. The eluate is then withdrawn and centrifuged for removal of acrylamide residues for five minutes at 12,000 rpm. The supernatant is then precipitated.

2.1.9 Ligation of DNA Fragments

In a ligation reaction, DNA fragments are coupled with complementary, single-stranded overhanging ends or with double-stranded blunt ends using T4 DNA ligase. This enzyme catalyzes, under ATP consumption, esterification between a 3' hydroxyl group and a 5'

phosphate group. Vector and DNA fragment to be inserted ("insert") are used in this reaction in roughly the ratio 1 : 3.

The ligation preparation includes, in addition to the vector- and fragment-DNA, 1 U T4 DNA ligase (GIBCO/BRL, Eggenstein) in a total volume of 20 to 50 μl (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% PEG 8000; pH 7.6). The preparation is incubated for 2 to 20 hours at room temperature or at 16°C. One-half of the preparation is used for the bacteria transformation.

2.1.10 Bacteria Transformation

Transformation refers to the introduction of plasmid DNA into bacterial cells, whereby their genetic properties (e.g., antibiotic resistance) can be altered. The introduction of DNA can occur by means of the $CaCl_2$ method or by electroporation.

2.1.10.1 $CaCl_2$ Transformation

For the transfer of recombinant plasmid DNA into *E.coli* cells, they must be prepared, i.e., made competent. By treating the bacterial cells with an excess of calcium ions (Dagert & Ehrlich, 1979), the membrane of the cells is altered such that the intake of DNA is rendered significantly easier. For this, 100 ml of a bacterial culture is incubated at 37°C in the shaker to an optical density of 0.3 to 0.4. After a 10-minute incubation on ice, the bacteria are centrifuged out for 5 minutes at 5000 rpm (Sorvall RC-5B, GSA-Rotor) and mixed into 20 ml of ice-cold 0.1 M $CaCl_2$. This cell suspension remains on ice for an additional 20 minutes. After recentrifugation, the bacteria sediment is added to 1 ml ice-cold 0.1 M $CaCl_2$ and incubated for at least 1 hour on ice. One-half of a ligation preparation that is to be introduced into the bacterial cells is added to 100 μl each of competent cells. As a negative control, preparations with distilled water are used; a defined quantity of circular plasmid DNA is used as a positive control. For the absorption of the DNA, the transformation preparation is subjected to a rapid temperature change (15 min./ice; 5 min./37°C; 15 min. ice; 5 min. ice/37°C; 15 min./ice). The preparation is then transferred to 0.5 ml LB medium and

incubated for the expression of the selection gene for 30 to 60 minutes at 37°C in the shaker. The transformation preparations are then spread on selective agar plates and incubated for 12 to 24 hours in the incubator at 37°C. Only the bacterial cells which have acquired antibiotic resistance by absorption of the foreign DNA can now be grown to form colonies and to enrich the foreign DNA in the process.

2.1.10.2 Electro-Transformation

Through electroporation, bacterial cells are forced, under the action of very high field intensities (10 kV/cm), to absorb foreign DNA. Because of the high field intensities, pores through which the DNA can penetrate form in the membranes of the bacterial cells.

500 ml of bacteria culture is cooled on ice for 10 minutes at an OD_{600} of 0.3. In the following process steps, the cells must be kept cold. The cells are sedimented (15 min./5000 rpm, Sorvall refrigerated centrifuge, GSA-Rotor) and washed in 500 ml ice-cold *Aqua bidest.* This procedure is repeated until complete elimination of salt residues from the growth medium. The bacterial sediment is then resuspended in 50 ml of 15% glycerol and transferred into 50 ml Greiner tubes and sedimented. The sediment is resuspended in 1.5 volumes of 15% glycerol and stored in 100 μl portions at -70°C.

The prepared, competent cells are thawed. The DNA precooled in Eppendorf vessels is thoroughly mixed with 50 μl of cells and transferred into precooled cuvettes (20 mm interelectrode distance). After the adjustment of the device (Gene Pulser to 2.5 kV and 25 μF, Pulse Controller to 400 ohm), electroporation takes place by producing a contact between the electrodes of the cuvette in a slide provided for that purpose. The pulse lasts 9 to 10 msec. Immediately after the pulse, the cells are transferred to 0.5 ml LB medium, incubated at 37°C for from 40 to 60 min. in the shaker, spread on selective agar plates, and incubated overnight at 37°C in the incubator.

2.1.11 Characterization of Recombinant Plasmid DNA by Restriction Cleavage

Using restriction analysis, the plasmid DNA, which was obtained after the bacteria transformation and subsequent plasmid isolation, can be checked for its proper structure.

A so-called reverse cleavage takes place with the same restriction endonucleases that were used for the preparation of the recombined DNA fragments. The reverse cleavage again releases the vector fragments and foreign fragments inserted and thus enables verification of the cloning limits. Reverse cleavage is, however, not possible if two different restriction endonucleases with cocloneable ends were used, which then, after ligation, no longer contain a symmetric recognition sequence for one of the two endonucleases (e.g., *Nhe*I and *Xba*I).

A reversible cleavage with two restriction endonucleases which only cut in the vector but not in the integrated fragment, gives information as to whether the insertion fragment has been ligated in only once or more than once.

An orienting cleavage is performed when the inserted fragment bears identical ends, i.e., when the fragment ligated in can be incorporated in both possible orientations. For this, an asymmetric cut within the insert and one or more cuts in the vector are used, and fragments of differentiable size are obtained. The orientation of the inserted fragment can thus be unequivocally determined.

2.1.12 DNA Sequencing

The sequencing method developed by Sanger *et al.* (1977) enables determination of the nucleotide sequence of a specific DNA segment. For this, the plasmid on which the sequence of interest is located is denatured to individual strands. By addition of a primer oligonucleotide that specifically binds in the vicinity of the 5' region of the sequence, the starting point for the opposing strand synthesis is specified. The enzyme involved, a T7 DNA polymerase also incorporates, in addition to the normal four deoxynucleotide triphosphates

(dNTP) contained in the reaction preparation, the individual dideoxynucleotide triphosphate (ddNTP) present in lower concentration in each case. By the incorporation of one such ddNTP each in the four parallel synthesis reactions, because of the missing 3' hydroxyl group, further lengthening of the synthesized opposing strand is prevented, and there is thus a termination of synthesis at this point. Due to the termination events distributed statistically over the opposing strand sequence in all four nucleotide-specific reactions, DNA fragments of all possible chain links develop, which can subsequently be separated electrophoretically for the four reaction preparations into four parallel tracks.

2.1.12.1 Sequencing Reaction

The sequencing reaction is performed according to the Sanger method with the "T7Sequencing ™ Kit" according to the manufacturer's instruction (Pharmacia, Uppsala). 1.5 μg double-strand DNA are denatured in 0.1 M NaOH. After ethanol precipitation, it is dried and mixed in 10 μl H_2O. To this preparation, 2 μl "Universal Primer" or "Reverse Primer" (2 mM) and 2 μl annealing buffer are added. In the next 20 minutes at 37°C and an additional 10 minutes at room temperature, the corresponding oligonucleotide can hybridize with the single-stranded DNA. The radioactive marking is accomplished by the addition of 1 μl of water, 3 μl "Labeling Mixed-dATP", 1 μl α ^{32}P-dATP (10 μCi), 2 μl T7 DNA polymerase (1.5 U/μl) and incubation for 5 minutes at 37°C. 4.5 μl each of this reaction is added to 2.5 μl each of the so-called A,C,G,T-specific reaction preparations with the ddNTPs indicated. This 5-minute-long termination reaction results in termination of the sequencing reaction such that in the reaction preparation, after denaturing, each single-stranded DNA ends with a specific ddNTP. After termination of the reaction, the specimens are reacted with one volume of staining buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured for 5 minutes at 80°C, and loaded on a 6% denaturing acrylamide gel.

2.1.12.2 Production of a Sequence Gel and Gel Run

Sequence gels enable separation of the sequencing preparations. 6% vertical polyacrylamide gels with a thickness of 0.4 mm are used. To produce a sequence gel, 18 ml Rotiphorese sequencing gel concentrate, 49.5 ml Rotiphorese sequencing gel diluting medium, 7.4 ml Rotiphorese sequencing gel buffer concentrate (Roth, Karlsruhe), 600 µl AMPS (Serva, Heidelberg), and 30 µl TEMED (Serva, Heidelberg) are mixed. The smaller plate, from which the gel is to be released, is treated with acrylase (Stratagene, Heidelberg); the larger plate, on which the gel is to remain adhered after separation of the plates, is treated with Bind-Silane solution (5 ml EtOH, 100 µl HOAc, 5 µl Bind-Silane, LKB, Munich). After 1 to 3 hours, the gel has fully polymerized and is clamped into a vertical electrophoresis apparatus. The sequence gels are run at a constant power of 56 W with TBE buffer (0.5 M Tris/boric acid; pH 8.3; 10 mM EDTA) in a 1-hour pre-run. After loading of the specimens, the primary run takes place for from 2 to 5 hours at 1000 to 1300 V at approximately 30 mA. Then, the gel adhering to Bind-Silane is fixed in 10% acetic acid, the carbamide carefully washed out under running water, and the gel dried at 80°C in the drying oven. The subsequent exposure of an x-ray film (Fuji Medical X-Ray-Film, RX) takes place overnight at 4°C. After development of the film, the DNA sequence can be read from the film.

2.1.13 Polymerase Chain Reaction

The Polymerase chain reaction (PCR) is a method for amplifying small quantities of DNA *in vitro* (Mullis *et al.*, 1986). This reaction includes essentially three individual steps, which are repeated several times: Denaturation of double-stranded DNA, addition of short primers on the strand and opposing strand as well as a DNA synthesis reaction for chain extension. In addition to the detection of extremely small quantities of DNA, the polymerase chain reaction also permits selective modification of the DNA molecule by appropriate choice of the nucleotide sequence of an oligonucleotide.

2.1.13.1 Purification of the Oligonucleotides

The oligonucleotides used are present after their synthesis in a concentrated ammoniacal solution. To convert the oligonucleotides into aqueous solution, 360 µl of the oligonucleotide solution is precipitated by addition of 40 µl sodium acetate and 1.2 ml 96% ethanol. The oligonucleotide precipitate obtained is washed with 80% ethanol, dried, and mixed in water. The concentration of the oligonucleotide solution is determined photometrically and calculated according to the formula c(oligo) = O. D. × 33 [µg/ml].

2.1.13.2 Reaction Conditions

The PCR reactions are carried out in a total volume of 100 µl. A typical preparation includes, in addition to the DNA template, the following components:

- 200 µM each dATP, dCTP, dGTP, and dTTP
- 100 [translator note: possibly corrected manually to 10] µM each of the two primer oligonucleotides
- 2 mM $MgCl_2$
- 5 µl 20x reaction buffer
- 5 U *Tfl*-polymerase (Biozym)

The preparations are overlain with 70 µl mineral oil. The PCR reaction is performed using the "Thermal Cycler" (Perkin Elmer Cetus, Norwalk, USA). Denaturation usually takes place for 60 seconds at 92°C; oligonucleotide hybridization for from 30 to 45 seconds at 4 to 10°C below the specific melting temperature. The melting temperature of oligonucleotides is determined by the following calculation: Tm = N(A,T) × 2 + M(C,G) × 4, where N indicates the number of A and T nucleotides and M the number of C,G components. The chain extension reaction is performed, depending on the fragment length anticipated, between 1 (ca. 500 bp) and 4 (ca. 2000 bp) minutes. The 25 to 30 cycles are concluded by 5 minutes incubation at 72°C. For analysis, 5 to 10 µl of the 100 µl volume is subjected to agarose gel electrophoresis.

2.2 RNA Methods

2.2.1 Isolation of RNA from Eukaryotic Cells

A modified guanidinium isothiocyanate extraction was used for the isolation of cellular RNA. For this, the "RNeasy-Kit" of the company Qiagen was used. Since, with this method, no time and labor consuming centrifugation steps are necessary, several specimens can be processed in parallel.

The cells (10^6-10^7) are lysed by the addition of 350 μl of guanidinium isothiocyanate-containing buffer (RLT). The lysate is centrifuged at 12,000 rpm for 3 minutes. The supernatant is transferred into a new Eppendorf vessel and thoroughly mixed with 350 μl ethanol (70%). This mixture is applied to the "RNeasy" column. After 15 seconds of centrifugation at 8000 g, the RNA binds on the silica matrix. After the first washing step with 700 μl of the buffer RW1, washing is performed twice with 500 μl of a buffer (RPE) reacted with four volumes ethanol (96%). With the last washing step, centrifugation is performed for 2 minutes at 12,000 rpm, so the silica membrane is dried. The elution of the RNA is performed by addition of from 30 to 50 μl of water and centrifugation for 60 seconds at 12,000 rpm.

2.2.2 Examination of Cellular RNA by Gel Electrophoresis

The isolated RNA can be separated electrophoretically in denaturing, i.e., formaldehyde-containing agarose gels.

1.2 g agarose is brought to a boil in 84 ml water and 10x gel buffer (0.2 M MOPS; 50 mM Na acetate; 10 mM EDTA; adjusted with NaOH to pH 8.0) and cooled to 60°C. After addition of 6 ml formaldehyde, a flatbed gel is cast. This is coated with run buffer (see gel buffer, adjusted to pH 7.0). The RNA specimens to be analyzed are reacted in each case with the same volume of specimen buffer (66% formamide; 1x gel buffer; 8% formaldehyde; 0.1% xylene cyanol FF; 0.1% bromophenol blue; 1.1 mg/ml ethidium bromide) and denatured for 5 minutes at 65°C. The specimens are then kept on ice until loading the gel.

Electrophoresis takes place for from 4 to 5 hours at roughly 80 volt. The gel can be photographed directly, since ethidium bromide is contained in the specimen buffer. An RNA mixture that has RNA fragments of the following sizes is used as a size standard: 1.77 kb, 1.52 kb, 1.28 kb, 0.78 kb, 0.53 kb, 0.4 kb, 0.28 kb, 0.155 kb (GIBCO/BRL, Eggenstein).

2.2.3 RT-PCR [reverse transcriptase-polymerase chain reaction]

The detection of small quantities of RNA is possible in this way with two reaction steps. First, the RNA template is transcribed into DNA using a reverse transcriptase. Then, it is possible to perform a polymerase chain reaction with the (single-stranded) synthesized DNA.

After isolation of all DNA and uptake in 40 μl of water, 5 μl is used for the reverse transcription reaction. A total volume of 20 μl (10 mM Tris; 50 mM KCl; pH 8.3) contains the following components:

- 5 mM $MgCl_2$
- 375 μM dATP, dCTP, dGTP, and dTTP
- 1.5 μM of the primers
- 5 U StrataScript (Stratagene)

The reaction was performed for 60 minutes at 41°C. After 5 minutes at 92°C, 5 μl of this preparation was used for the PCR reaction (see 2.1.13).

2.3 Protein Methods

2.3.1 Expression of Proteins in *E.Coli*

Various expression vectors are available for the synthesis of the proteins in bacteria cells. The final concentration of a newly formed protein depends on the copy number of the plasmid, the strength of the promoter, as well as the ribosome binding site. To obtain the highest possible yield of a recombinant protein, a "*high copy*" plasmid vector was used that has the strong p_LO_L promoter of the bacteriophage λ (see Fig. 2-1). In *E.coli* cells that express a cI857 repressor, the initiation of the transcription can be thermally regulated. At 32°C, the repressor binds to the O_L operator sequence and prevents the transcription of the

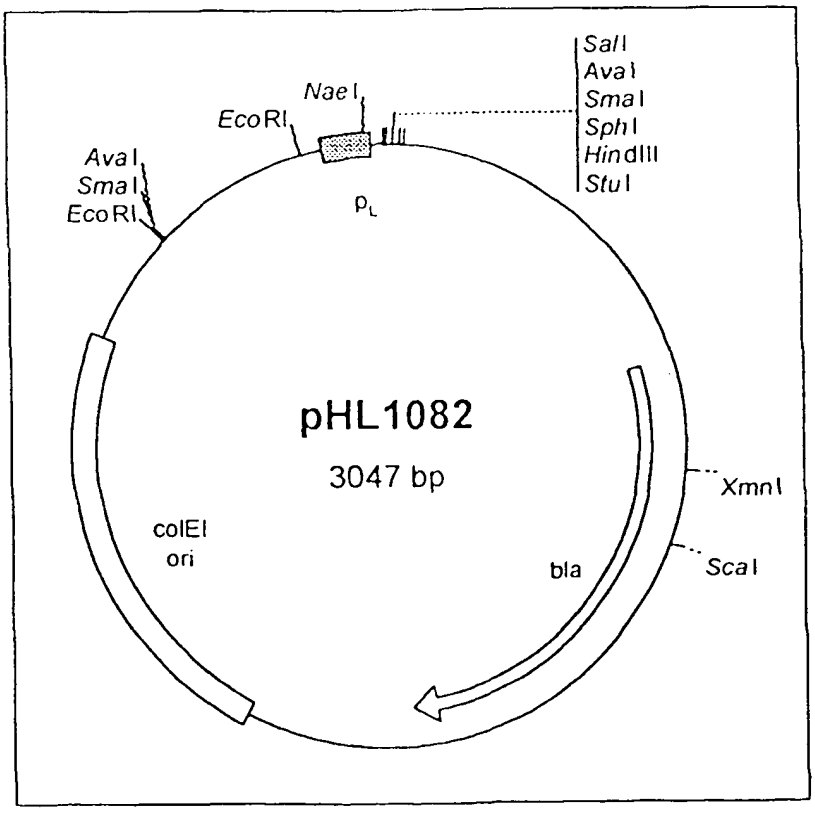


Fig. 2-1: Structure of the expression vector pHL1082
The plasmid pHL1082 contains the p_L promoter and operator O_L of the bacteriophage λ from the vector pEMBLex2 (Solazzo *et al.*, 1985). This permits regulatable protein expression in *E.coli* cells, in which the mutant cI857 of the λ-repressor is present. For the amplification in *E.coli* cells to high copy numbers, a corresponding plasmid origin of replication (colEI-ori) is used. Ampicillin resistance is mediated by beta-lactamase (bla).

promoter p_L. After increasing the temperature to 42°C, the repressor is inactivated by conformation change and leaves the operator region. Now the transcription can take place from the strong p_L promoter. For the efficient initiation of the translation, the construct includes the Shine-Dalgarno sequence from the MS2 replicase gene. For the synthesis of the temperature-sensitive repressor mutants, the *E.coli* strain designated as GC382 was used with the plasmid pcI857 that enables expression of the repressor molecule (Remault *et al.*, 1983).

2.3.2 Purification by Means of Affinity Chromatography

Recombinant proteins that have six histidines ("his-tag") on their N- or C-terminus can be purified using metal chelate affinity chromatography. (Hochuli *et al.*, 1987). The column material (Ni-NTA, Qiagen) is made of nitrilotriacetate groups covalently bound to Sepharose as well as of complex-bound nickel ions at four sites. In each case, two of the histidine residues of the fusion protein bind to the remaining two octahedral sites of the nickel and thus form a stable complex bond (Kd = 10^{-13}) with the Ni^{2+} ions. The nonspecifically bound proteins can be washed out such that after elution with imidazole (or lowering of the pH value) highly purified protein fractions are obtained.

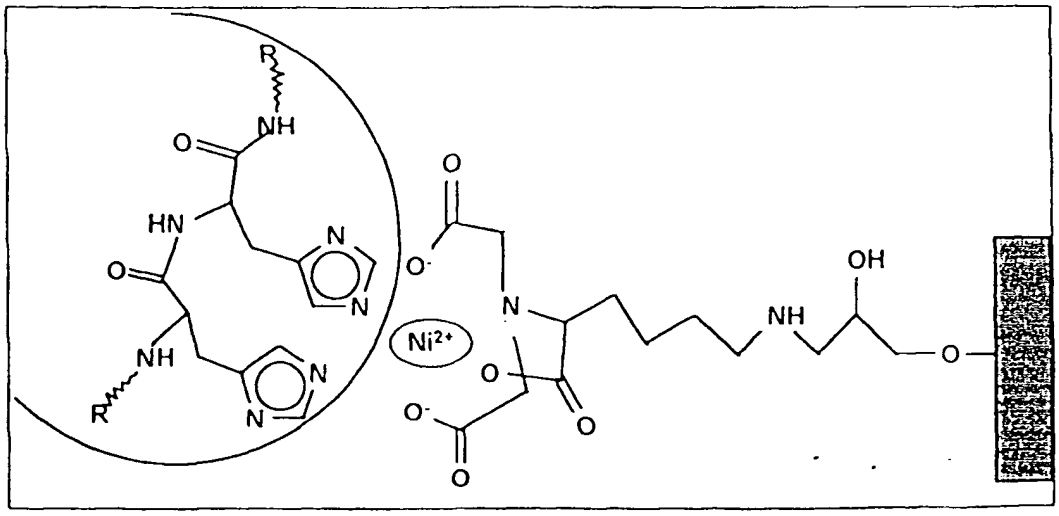


Fig. 2-2: Complex bond between histidine residues and nickel-nitrilotriacetate
The Ni^{2+} ion with a coordination number of 6 can form four bonds with the nitrilotriacetate bound on Sepharose. Two ligand sites can be occupied by the histidine residues of recombinant proteins. After binding of the proteins, the complex bond can be competitively broken by the addition of imidazole and the bound protein thus eluted.

Proteins soluble in the cytoplasm of the bacterial cells can be naturally purified, i.e., under physiological conditions the tertiary structure of proteins is retained such that the purified protein fractions can be used directly for functional analyses (such as enzymatic reactions or DNA binding reactions).

For the protein expression, 5 ml of an overnight culture are used to inoculate 500 ml of a new culture. The bacteria culture is incubated at 30°C until it has reached an OD_{600} of from 0.7 to 0.8. Then, the temperature is raised to 42°C and the culture incubated for from 5 to 7 hours. The bacteria are then harvested by centrifugation.

The bacteria sediment (1 to 3 g) obtained after centrifugation is mixed in from 20 to 40 ml sonication buffer (10 mM Tris-HCl pH 7.8, 50 mM KH_2PO_4; 300 mM NaCl; 10 mM β-mercaptoethanol) and sonicated under cooling on ice at 80 W. The cellular debris is centrifuged out in a Sorvall refrigerated centrifuge at 14,000 rpm for 30 minutes at 4°C.

The column coated with 4 ml of column material is first equilibrated with 20 ml sonication buffer. Then, the supernatant is applied to the column. After that, a large share of the nonspecifically bound bacterial protein is removed by the addition of 20 ml washing buffer (40 mM Tris-HCl pH 7.5; 20% [v/v] glycerol; 100 mM KCl; 1 mM β-mercaptoethanol). The protein is eluted by the addition of 4 ml each 50/80/120/200/300/500 mM imidazole in washing buffer. Fractions of one ml each are collected in Eppendorf vessels and an aliquot separated on an SDS polyacrylamide gel. The composition of the protein fractions as well as the quantity of recombinant protein can be determined after Coomassie staining.

2.3.3 SDS Polyacrylamide Gel Electrophoresis

SDS gel electrophoresis is a method for separation of proteins of differing molecular weight over a discontinuous polyacrylamide gel. SDS results through hydrophobic interaction of its carbon chain with the hydrophobic portions of the proteins to break up the tertiary structure. This results of the formation of a polyanion, whose surface charge is proportional to molecular size. Thus, size determination is possible after gel electrophoresis and comparison with known control proteins (Laemmli *et al.*, 1970). The discontinuous gels consist of a separation gel and a collection gel with a pH differential, which results at the pH jump in a "collection" of the protein molecules and, by means of this focusing, in improved separation of the proteins.

First, a separation gel is cast. The concentration of the gel matrix is selected on the basis of protein sizes of interest. The separation gel buffer consists of an aqueous solution with 1.5 M Tris-HCl pH 8.8 and 0.4% SDS.

To form a sharp boundary layer, the separation gel is coated with water after casting. After complete polymerization of the separation gel, the water is removed and the collection gel cast directly on the separation gel. The collection gel solution is made up of the following components: 0.65 ml 30% AA/bisAA; 3.05 ml aqua bidest; 1.25 ml loading gel buffer (0.5 M Tris-HCl pH 6.8; 0.4% SDS); 25 μl AMPS; 5 μl TEMED). An aqueous solution with 25 mM Tris-HCl, 0.2 M glycine, and 0.1% SDS is used as an electrode buffer.

Before loading, the specimens are incubated for 5 minutes at 90°C in SDS specimen buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol; 1 mM EDTA; 0.005% bromophenol blue, 62.5 mM Tris; pH 6.8). The protein gels are run with a constant voltage of 150 volts until the dye front has moved with the BPB out of the loading buffer to the lower edge of the gel.

For determination of the protein size, a molecular weight standard marker from the company Promega was used, consisting of the following proteins:

Protein	Molecular Weight [kDa]
Bovine serum albumin	97.4
Phosphorylase B	66.2
L-glutamine dehydrogenase	55.0
Ovalbumin	42.7
Aldolase	40.0
Carbon anhydrase	31.0
Soybean trypsin inhibitor	21.5
Lysozyme	14.3

The gels are stained (20 minutes) with a staining solution (9% glacial acetic acid, 45% methanol, 0.1% Coomassie brilliant blue), destained (3 × 20 minutes in 50% methanol, 7.5% glacial acetic acid), and dried.

2.3.4 BCA Protein Determination

The protein quantity can be determined using an off-the-shelf test process according to the manufacturer's instructions (Pierce). The protein preparation as well as various diluents from BSA are incubated in alkaline solution (0.4 mM NaOH, 4% $CuSO_4$) for 30 minutes at 60°C with BCA (bicinchoninic acid). Under these reaction conditions proteins Cu^{2+} oxidize to Cu^{+}. The monovalent copper ions form a water-soluble complex with the BCA that has an absorption maximum at 562 nm. After establishing the calibration curve with various concentrations of BSA (0 to 20 μg), the protein concentration can be determined.

2.3.5 Western Blot

Western immunoblots were performed for the identification of proteins. For this, the proteins are separated in SDS polyacrylamide gel and transferred to a nitrocellulose filter according to standard methods (Maniatis *et al.*, 1989). The blot is performed by means of transfer buffer (192 mM glycine, 25 mM Tris, 5% methanol) at 4°C overnight at 50 mA. Then, the NC filter is stained with a dye solution (2% Ponceau S, 3% trichloroacetic acid) to check the transfer. To neutralize nonspecific binding sites, the filter is placed for 1 hour in NET buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.25% gelatin, 0.05% Triton-X-100, 2% BSA). After washing with 0.1% Tween 20 in PBSminus (3 × 10 min) and H_2O (1 × 10 min), the filter is incubated with the specific antiserum directed against the protein to be detected (diluted 1 : 200 in NET buffer). After incubation with protein A-peroxidase conjugate (1 μg/ml in 20 ml NET buffer) for 60 minutes, the nitrocellulose filter is incubated for 10 minutes with diaminobenzidine (0.5 mg/ml in 100 ml PBSminus). After addition of 2 μl H_2O_2 (30% w/v), the diaminobenzidine is oxidized by the bound peroxidase, and brown bands become visible. The reaction is terminated by washing with water. After drying, the filter is stored protected from light to prevent fading.

2.3.6 Immunofluorescence

Expressed proteins can be identified using incubation of cells with a specific antibody. By subsequent incubation with a second antibody marked with a fluorescing dye (fluorescein), the binding of the first antibody can be visualized after UV irradiation.

The cells are washed three times with PBS and then fixed with an acetone/methanol mixture (1 : 1) for 10 to 15 minutes at -20°C. After drying, the first antibody (monoclonal antibody

clone 13/45/31, dianova, Hamburg) is diluted 1 : 50 in PBS. This is followed by binding at 4°C for 5 to 12 hours. The cells are washed once with PBS. This is followed by incubation with the second antibody (1 : 50 in PBS) at 37°C for 1 hour. After washing with PBS, the fluorescing cells are observed or documented photographically under the fluorescent microscope.

2.4 Cell Culture

2.4.1 Cell Lines and Media

For the transfection and infection experiments, the following cell lines were used:

- B82: mice fibroblasts
- MDCK: canine kidney cells
- COS-7: monkey kidney cells
- Vero: monkey kidney cells
- HeLa: human carcinoma cells
- SKNM660: Human oligodendrocytes

The permanent cell lines used were cultured at 37°C in an incubator (Heraeus, Labotect: Steri-Cult 200 Incubator) at 96% humidity and a 5% CO2 concentration. *Eagle's minimal essential medium*, modified according to Dulbecco (DMEM) is the culture medium used. The medium also contains 10% [v/v] fetal calf serum (FKS; [English = FCS]) as well as 1% [v/v] antibiotic/antimycotic (GIBCO/BRL). At regular intervals, the cells grown confluently in Petri dishes are thinned. For this, the cells are washed with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4) and overlayered with a PBS solution that contains 0.2% [w/v] trypsin and 0.02% [w/w] EDTA. After detachment of the cells from the Petri dish (depending on the cell line 1 to 30 minutes), the reaction is stopped by the addition of the same volume of DMEM culture medium (trypsin inactivation by serum proteins). An aliquot of the detached cells is then divided among new Petri dishes and incubated at 37°C in the incubator.

2.4.2 Virus Strains

The avian virus strain A/FPV/Bratislava (generously made available by P. Staeheli, Freiburg) has multiple advantages for the transfection/infection experiments performed. Since it is a variant adapted on mouse cells, virus replication on this cell type is very high. After 8 hours, a replication round ends, such that the virus can be applied to new cells. Trypsin inactivation is not necessary since the strain is of the subtype H7 and, consequently, is also cleaved by ubiquitous cellular proteases such as furin. This facilitates comparability of the individual experiments since a secondary effect that can be caused by a different extent of trypsin cleavage is precluded. The strains A/Asia/57 (H2/N2) as well as A/Victoria/3/75 (H3N2) are used to test whether the results obtained for the avian virus also apply as a model for human strains.

2.4.3 Virus Replication

It is possible to replicate influenza A virus strains in cell lines (e.g., MDCK) or in 11-day-old embryonated hens' eggs (Burnett & Stone, 1940).

For this, an aliquot of the virus suspension is diluted depending on the virus titer in PBS(+) 1: 1000 to 1 : 10000. The embryonated hens' eggs are disinfected on the blunt pole with tincture of iodine and punctured with a steel needle. 0.2 ml of the dilute virus suspension is injected vertically through the air chamber into the allantoic cavity, and then the injection site is sealed with wood glue (Ponal, Henkel). The eggs are incubated for 48 to 72 hours at 37°C and candled twice daily during that time. Eggs which contain dead embryos within the first 24 hours are discarded. Eggs with embryos that died later are kept at 4°C. All eggs are incubated at 4°C at least 8 hours before the removal of the allantoic fluid in order to effect a constriction of the blood vessels. For the removal of the allantoic fluid, the eggs are opened above the air chamber, the chorioallantoic membrane punctured, and the fluid withdrawn by pipette. With this process, titers of from 10^7 to 10^{10} infectious viruses per ml are obtained.

2.4.4 Hemagglutination Test

Influenza viruses have the capability, through multiple binding of their surface protein hemagglutinin, to cross-link erythrocytes of specific species. This hemagglutination is utilized and the titer of hemagglutinating virus particles is determined using a quickly performable hemagglutination test (HA test) (Hirst, 1941).

For the HA test, 50 μl of a 0.9% NaCl solution is first placed in a microtiter plate. An equal volume of the virus suspension to be tested is added and the mixture continuously diluted logarithmically, base 2. By addition of a hen erythrocyte suspension (1%), it is possible to evaluate the sedimentation pattern after 30 minutes. If the concentration of the hemagglutinating virus particles is too low, there is no agglutination and the erythrocytes sediment into sharply delimited complexes. The HA titer is calculated from the reciprocal of the last dilution at which agglutination is still observed. A HA titer of 1 corresponds to roughly 106 virus particles per ml. However, the HA test detects both infectious and noninfectious virus particles, and a concentration determination of the infectious viruses for their share in the total virus in this manner is not possible.

2.4.5 Plaque Test

In contrast to the HA test, using the plaque test, it is possible to determine the number of infectious virus particles of an egg fluid or of a cell supernatant (Zimmermann & Schäfer, 1960). After successful infection of an individual cell with a virus particle, the virus progeny are prevented from free diffusion by overlayering with semisolid agar and can thus infect only adjacent cells. By lysis of these infected cells, holes (plaques) develop in the cell lawn, which, by counting and evaluation, yield the infectious titer.

First, the confluently grown MDCK cells are washed with PBS+. The virus suspension to be tested is first diluted in steps of 1 : 10 each from 10^0 to 10^{-6} in PBS+. 500 μl of each dilution step are placed on one cell plate each, previously washed with PBS+. For the adsorption of

the viruses on the cell surface, the cells are incubated from 30 to 60 minutes at 37°C. Then, the cells are washed with PBS+ and overlayered with 42°C-warm plaque test agar (1 : 1 mixture of 2x DMEM without FCS and 1.9% agar). After setting of the agar, the incubation of the infected cells is carried out for from 2 to 4 days at 37°C. The number of plaques formed corresponds to the number of infectious viruses and is reported under consideration of the dilution sequence in PBE [German], i.e., PFU, per ml (plaque forming units) (Dulbecco, 1952).

2.4.6 Lipofectamine DNA Transfection

Many methods are available for the introduction of plasmid DNA into eukaryotic cells. One process with a very high transfection yield is LipofectAMINE transfection. LipofektAMIN™ Reagent (GIBCO/BRL, Eggenstein) contains the substances DOSPA (a polycationic lipid) and DOPE (a neutral lipid) in a ratio of 3 : 1 (w/w). These components form positively charged liposomes, which form complexes with negatively charged DNA, and fuse with the cell membrane with their hydrophobic component. In order to obtain optimum transfection efficiency, various parameters, such as lipofectamine/DNA ratio and lipofectamine/cell (type, density, number), must be varied. Moreover, the transcription system (RNA polymerase I or RNA polymerase II) of the recombinant plasmid used is a significant factor for the level of the expression of the genetic information introduced.

For the DNA transfection of B82 cells with plasmid DNA, which contain the murine polymerase I transcription system, 10^7 B82 cells at 70- to 80% confluence are used. The cells are washed twice with a serum-free medium. In a polystyrene tube, 5 μg plasmid DNA is mixed with 30 μl LipofektAMIN™ (1 μg/μl) and 370 μl serum-free DMEM medium and incubated for at least 15 minutes at room temperature. After addition of 4 ml DMEM medium (without FCS), the mixture is then applied to the cells. After a 1-hour incubation, the DNA/lipofectamine mixture is replaced by culture medium (DMEM medium with 10% FCS) and incubated for 24 hours in the incubator. During this incubation period, there is

fusion of the liposomes vesicles with the cell membrane, whereby the DNA arrives in the interior of the cell. Then, infection with influenza viruses occurs.

2.4.7 Primary Infection and Passage

The cells are washed twice with PBS+ and then incubated for from 45 to 60 minutes with virus-containing PBS (+) in the incubator. PBS with the bivalent ions contained therein serves to stabilize the influenza viruses during adsorption. The infected cells are washed again, and, depending on the virus type or CPE [cytopathic effect], are incubated for from 8 to 24 hours with DMEM medium (with 10% FCS). During this time, the replication of the viruses occurs.

After that, the cells are harvested for additional analyses and the virus-containing supernatant is purified of cell debris by centrifugation (1200 rpm, 5 min). An aliquot (usually 0.5 to 1 ml) of the supernatant is mixed with PBS (+) and then applied for 45 to 60 minutes to approximately 10^7 MDCK cells. After 8 to 24 hours, the cytopathic effect (CPE) is monitored; and both cells and supernatant are treated as described above for the primary infection.

2.5 Detection of Reporter Genes

Using enzyme reactions, the expression of foreign genes which were introduced into the cell after transfection or infection can be detected. Whereas the transcription activity of a gene can be detected by RNA analyses, the use of reporter genes enables conclusions concerning the efficiency of the protein expression.

2.5.1 CAT Analysis

A frequently used reporter gene is chloramphenicol acetyltransferase (CAT) originating in *E.coli*. This enzyme catalyzes the transfer of the acetyl group from acetyl coenzyme A to chloramphenicol (see Fig. 2-3). The reaction products can be separated by thin film chromatography. If fluorescently-labeled chloramphenicol is used as the substrate for the CAT reaction, the separated products can be visualized under long wave UV light (366 nm) and the reaction yields can thus be measured.

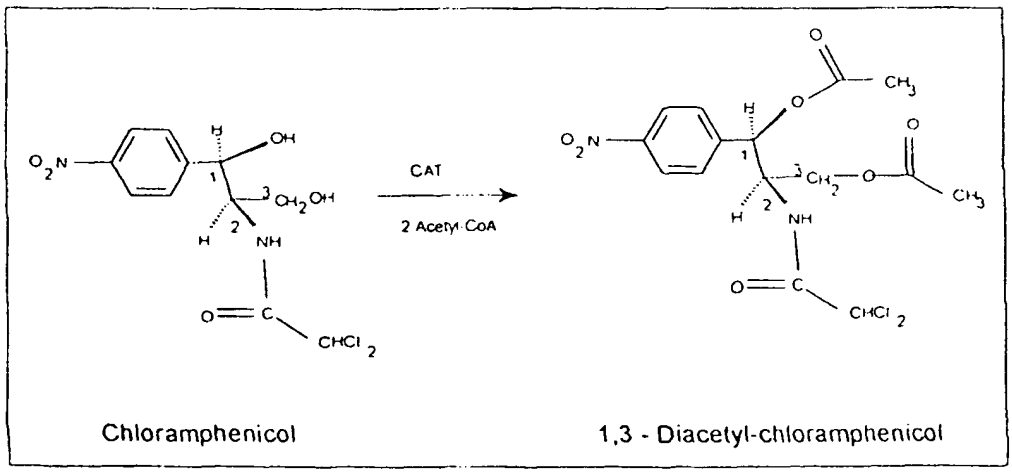


Fig. 2-3: Chloramphenicol-Acetyl-Transferase (CAT) Reaction
Chloramphenicol acetyltransferase (CAT) originating in *E.coli* catalyzes the transfer of the acetyl group from acetyl coenzyme A to chloramphenicol. The chloramphenicol and the acetylation products 1 or 3-monoacetyl-chloramphenicol, as well as 1,3-diacetyl-chloramphenicol can be separated by thin-film chromatography.

The cells are washed once with PBS, detached from the plates after the addition of 1 ml of PBS using a rubber scraper, and transferred into Eppendorf vessels. The cells are then centrifuged for 5 minutes at 1600 rpm. The cell sediment thus obtained is resuspended in 110 µl Tris-HCl (0.25 M pH 7.5), and then the cell membranes are broken down by repeated temperature changes ("freeze and thaw"). In this process, the cell specimens are frozen twice each by immersion in liquid nitrogen and thawed by incubation in the 37°C waterbath for 5 minutes in each case. Then, the preparations are incubated at 56°C for 10 minutes to destroy any deacetylases present, which would catalyze the disintegration of the acetylated forms. The cell debris is sedimented by a 10-minute centrifugation at 12,000 rpm. The cell lysates contained in the supernatant can then be used for the determination of the protein concentration (by BCA assay) and for the enzyme reaction.

Florescence-marked chloramphenicol (borodipyrromethanedifluoride-fluorophore; FLASH-CAT-Kit, Stratagene) is used as the substrate for the CAT reaction. In an Eppendorf vessel, 30 to 50 µl cell lysate is mixed with 5 µl acetyl-coA (4 mM), 10 µl 0.25 M Tris/HCl (pH 7.5) and 10 µl florescence-marked chloramphenicol (BODIPY™ CAM Substrate) and incubated at 37°C. After 2 to 12 hours, 0.5 ml ethyl acetate is added for the extraction, and centrifugation is performed for 1 minute at 12,000 rpm for the phase separation. 450 µl of the upper phase is transferred into Eppendorf vessels and lyophilized (45 minutes in the vacuum centrifuge), mixed into 10 µl ethyl acetate, and applied to thin-film chromatography plates (TFC ready-to-use plates silica gel 60, 20 × 20 cm, film thickness 0.25 mm; Merck). An 87 : 13 chloroform: methanol mixture is used as the solvent for the chromatography. Then, under UV illumination, the separated reaction products can be documented photographically.

2.5.2 Beta-Galactosidase Assay

The beta-galactosidase enzyme isolated from *E.coli* cleaves the disaccharide lactose into glucose and galactose. If the substrate ONPG [o-nitrophenyl-beta-D-galactopyranoside] is used, the enzyme also cleaves this β-glycosidic bond producing o-nitrophenol. Its absorption is measured at 420 nm.

0.5 ml of an overnight culture is inoculated in 5 ml medium and agitated for 90 minutes at 37°C until OD_{600} = 1. The cultures are transferred into window gel tubes, cooled for 15 minutes on ice, and then centrifuged (10 minutes at 4000 rpm). The supernatant is discarded, the sediment is resuspended in 5 ml Z-buffer. Now, 1 ml of this suspension is transferred into a measuring cuvette for the measurement of the OD_{600}. The remaining 4 ml are sonicated (40 seconds at 80 W), whereby the breakdown of the bacterial cells occurs. The cell content and thus the β-galactosidase are now found in the sonicate. 1 ml is removed from this and transferred into a 2-ml Eppendorf vessel. The reaction is started by addition of 0.2 ml ONPG (4 mg/ml). The reaction is stopped by the addition of 0.5 ml NA_2CO_3 [sic] (1 M). A centrifugation step (10 minutes, 8000 rpm) takes place now for the removal of cell debris. 1 ml of the supernatant is transferred into a measuring cuvette to determine the OD_{420}. The activity of the β-galactosidase is calculated according to the following formula:

$$U_{\beta\text{-gal}} = \frac{OD_{420} \times 1000}{OD_{600} \times t_{min} \times vol}$$

OD_{420}: optical density at 420 nm
t_{min}: reaction time in minutes
vol: test volume
OD_{600}: optical density at 600 nm

2.5.3 Detection of the Expression of Green-Fluorescing Proteins by Fluorescence Microscopy and Flow Cytometry

The green fluorescing protein (GFP) originating from the jellyfish Aequorea victoria presents directly visible fluorescence without exogenous addition of substrate or cofactors. The chromophoric group forms autocatalytically from the tripeptide sequence Ser^{65}-Tyr^{66}-Gly^{67} (see Fig. 2-4). The wildtype GFP has two excitation maxima at wavelengths 396 nm and 475 nm; irradiation results in emission of a wavelength of 508 nm. The substitution of the serine residue at position 65 with a threonine results in suppression of the absorption maximum at 395 nm and intensification of the absorption at 470 to 490 nm. This "S65T" mutant is particularly well-suited, because of its increased florescence, for analysis with fluorescence microscopy

and FACS [fluorescence activated cell sorting] analysis, since both processes are already optimized for excitation at 480 nm and detection of green emission light because of the same

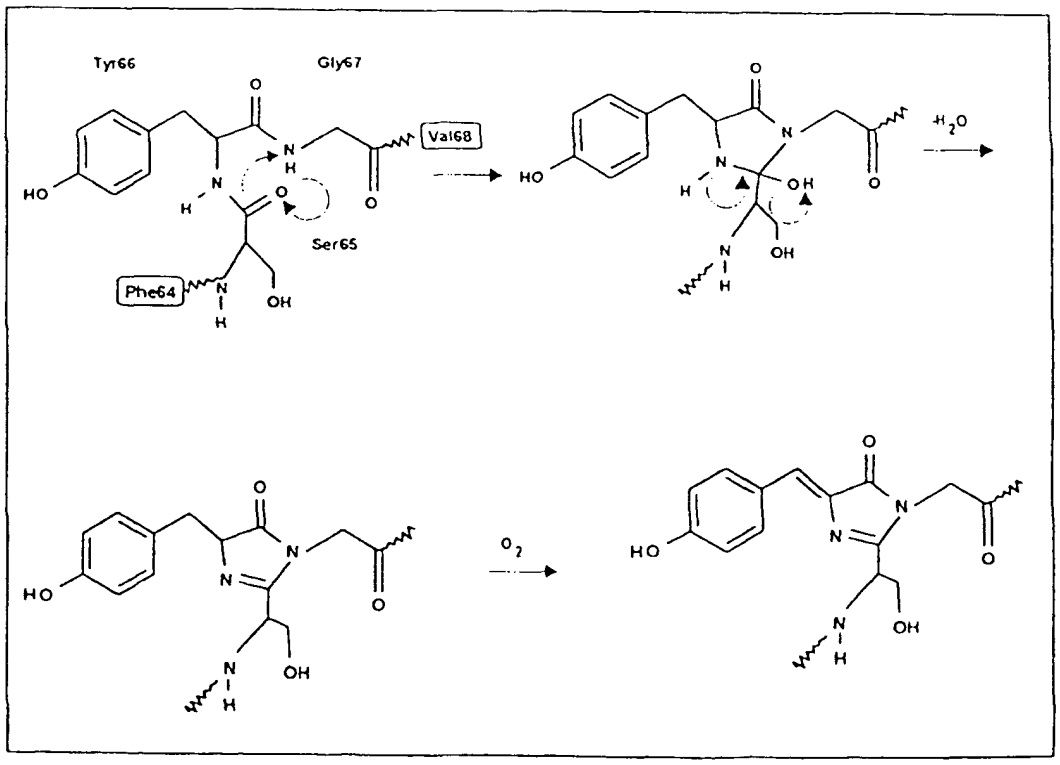


Fig. 2-4: **Reacti n Mechanism f r th Formation of Chromophoric Groups in GFP** (after Cubitt *et al.*, 1995) The fluorophoric group of the GFP forms autocatalytically from the tripeptide sequence Ser^{65}-Tyr^{66}-Gly^{67} within the polypeptide chain including 238 amino acid. First, there is cyclization with water cleavage. Under aerobic conditions, oxidation to a p-Hydroxybenzyliden-imidazolinone then occurs. This structure has two excitation maxima (396 nm and 475 nm), which results in fluorescence emission at a wavelength of 508 nm.

fluorescent absorption and fluorescent emission with the widely used FITC (fluorescein-isothiocyanate) in immunofluorescence, and also in the fluorescence-activated flow cytometry (FACS).

The Universal Axioplan Microscope (Zeiss, Jena) was used for the direct detection of fluorescing cells. For the reflected-light fluorescence examinations, the filter set "blue 450-490" (487909) with the filter combination for excitation filter "BP 450-490" (447722), color separator "FT 510" (446434), and suppression filter "LP 520" (447737) was used. For the documentation the microscope-camera MC100 was employed.

For the flow cytometry, the "FACSort" device from the company Becton Dickinson was used. Using this device, the fluorescence of a single cell can be detected. Excitation occurs using an argon laser that emits monochromatic light with the wavelength 488 nm. The filter FL1 was used for detection of the emitted light. After conversion of the light pulses into electrical signals by means of a photomultiplier and amplification of the signals, the data were evaluated using the software "CELLQuest".

3 RESULTS

With the methods of selective mutagenesis, it is possible to determine significant functional elements in the lifecycle of influenza viruses. Using the RNA-polymerase I transcription system, such mutation analyses can be performed *in vivo*. Here, with the plasmid pHL926 (Neumann, 1992) between the rDNA promoter and the rDNA terminator of the mouse directly adjacent, the noncoding end regions of the HA-cDNA are located in *antisense* orientation. The HA-encoding sequence was replaced by the reading frame of the reporter gene chloramphenicol acetyltransferase (CAT). The expression of the genetic information can thus be more readily detected after primary and secondary infection (Section 1.3).

3.1 Cotransfection of the RNA-Polymerase I Transcription System with a *lac*I-Fusion Protein

After transfection of construct pHL926 on B82 cells and subsequent infection with influenza viruses, this resulted in detectable CAT activity. The expression of the CAT activity was, however, slight in this case. The quantity of primary transcripts might not have been adequate, such that only a few vRNA molecules are available for viral amplification, which then results in limited protein expression. The transcription by the cellular RNA polymerase I occurs in the nucleolus. An improvement of the efficiency of the RNA polymerase I transcription system should, consequently, be achievable if the concentration of transfected plasmid molecules in the nucleolus is increased.

A higher copy number of plasmids inserted in the nucleolus might occur through improved transport from the cell nucleus into this subcompartment. A protein that can bind to the plasmid DNA and also has a nucleolus localization signal (NLS) should be able to mediate this transport. The *lac*I repressor isolated from *E.coli*, which binds specifically on the *lac*

operator sequence with a high binding constant, is a DNA-binding protein. For the *rex*-protein of HTLV I, a basic amino acid sequence necessary and sufficient for the transport of this protein into the nucleolus has been described (Siomo *et al.*, 1988; Nosaka *et al.*, 1989).

Consequently, the following idea was developed: A fusion protein consisting of the *lac*-repressor and a nucleus localization signal from *rex*-protein should be produced. For easier purification with affinity chromatography, this construct should contain six histidine residues. After the protein purification, the isolated protein is incubated along with a plasmid with *lac*-operator. The binding of the protein to the DNA should then, after liposome transfection of these complexes into the cell, increase the transport rate of the DNA molecule with the RNA polymerase I transcription system into the nucleolus. An improvement of the RNA polymerase I expression would then be measurable by CAT analysis after infection with influenza helper viruses.

The plasmid pHL1090 was constructed (See Fig. 3-1) for thermally regulatable expression in the *E.coli* strain GC382. It is based on the expression vector pHL1082 (Fig. 2-1) and includes

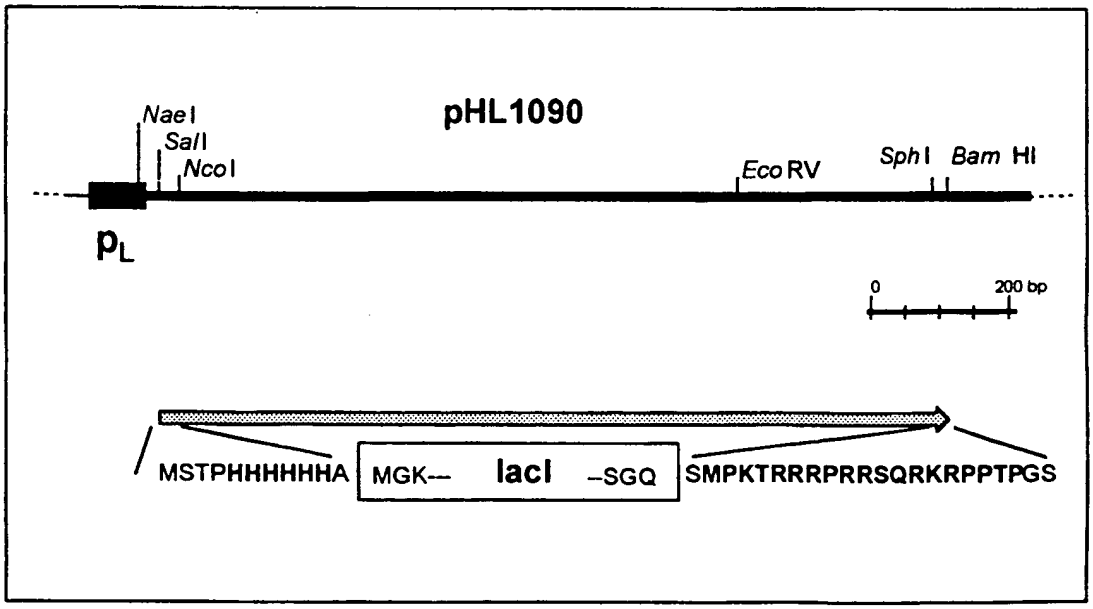


Fig. 3-1: Structure f the plasmid pHL1090 for th xpression of th *lacI* fusion protein
pHL1090 contains the p_L promoter and operator O_L of the bacteriophage λ. This permits the regulatable protein expression in *E.coli* cells in which the mutant c1857 of the λ-repressor is present.
The fusion protein has on its N-terminal end six histidine residues for easier purification by means of affinity chromatography. The nucleolus localization signal of the *rex*-protein of HTLV I was attached on the C-terminus.

the encoding sequence for the *lac*I fusion protein is composed as described. For purification by the affinity chromatography, the recombinant protein on the N-terminal end has six histidine residues. A polypeptide chain with 21 amino acids, which includes the nucleolus localization signal of the *rex*-protein of HTLV-I, was fused on the C-terminus.

In order to test whether the DNA-binding of the *lac*I protein is still possible after the joining of additional amino acids, an *in vivo* detection was established in *E.coli*. The detection system consists of three plasmid components selectable by different antibiotic resistances: the plasmid pcI857 mediates kanamycin resistance and delivers the temperature-sensitive λ-repressor molecule cI857; the ampicillin-resistant pHL1090 is used for the expression of the *lac*-repressor. And finally, the reporter plasmid pHL1134 includes, in addition to the p15A-replication origin, a tetracycline resistance gene as well as the reporter gene *lac*Z under the control of a p_{Lac} promoter.

After electroporation of pHL1090 and pHL1134in GC382 cells, the colonies obtained were inoculated into a medium with the three antibiotics (kanamycin, tetracycline, and ampicillin). As a control, the plasmid pHL1082, which has no *lac*I fusion protein cassette, was used instead of pHL1090. By preparation after alkaline lysis, the presence of the plasmids was verified. For the *lac*Z-test, preparations with IPTG and without IPTG were used in each case. The *lac*Z-test performed (see 2.5.2) showed that with pHL1082, no significant difference in the reporter gene expression could be detected (-IPTG 12590 U/+IPTG 13910 U). In contrast, with pHL1090, the *lac*Z-expression was increased after addition of IPTG (-IPTG 5090 U/+IPTG 17330 U). The lower Beta-galactosidase value without the addition of IPTG proves that the *lac*I-fusion protein was synthesized in the bacteria cells and binding occurred on the *lac*-operator sequence of the p_{Lac}-promoter, and thus resulted in a lower transcription activity of the gene. By means of this control test, it was guaranteed that after temperature induction a fusion protein that binds specifically on the *lac*-operator sequence is synthesized.

For the protein expression, GC382 cells that have the λ-cI857 repressor were used (see 2.3.1 and 2.3.2). After digestion of the cells from the temperature-induced culture and absorption of the proteins on the nickel chelate column, a buffer that is suitable for DNA protein bonds was used for the elution. After electrophoretic separation in the polyacrylamide gel, the composition of the fractions obtained was verified. In the fractions that were obtained after elution with 120, 200, and 300 mM imidazole, an approximately 42-kDa-sized protein with a purity of > 90% was present (see Fig. 3-2). In a control preparation, the protein expression and protein purification of GC382 cells that were transformed with pHL1082 was performed under the same conditions. Here, no protein bands could be detected in the corresponding size. The size of the isolated protein matches the anticipated size of the 398-amino-acid-long recombinant protein.

In the construction for the reporter plasmid that contains *lac*-operator sequences in the vector component, an *Ssp*I fragment including 300 bp from pinf 4/49 with the *lac*I-operator sequence was inserted in the vector pHL926 cleaved with *Eco*RV/*Nae* I.

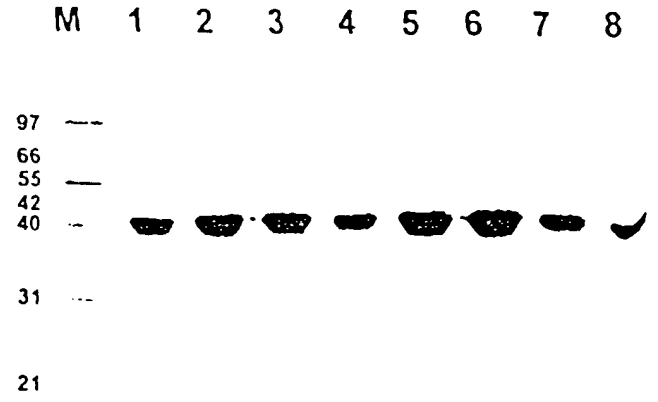


Fig. 3-2: Protein Purification of the Procaryotically Expressed lacI-Fusion Protein
After protein expression in GC382 cells, the cell lysates were placed on a Ni-chelate column. After a washing step, the bound proteins were eluted with an imidazole-containing buffer. An aliquot of the fractions obtained was separated on an SDS-polyacrylamide gel and stained with Coomassie brilliant blue.
In lane 1 through 4, the fractions obtained after elution with 120 mM imidazole are loaded; in lane 5 through 8, the fractions obtained after elution with 200 mM imidazole. The length marker is recorded in the lane labeled M; the sizes are indicated to the left of this lane in kDa.

The resultant plasmid was called pHL1122.

For the binding of the protein on the *lac*-operator sequence, different quantities (0, 2, 5 μg) of the protein were incubated with 5 μg plasmid DNA for 30 minutes at room temperature. The plasmid pSV2CAT that bears the CAT gene under the control of an SV40-promoter was used as a transfection control. For the formation of the liposome vesicles, the preparation was incubated for 15 minutes at room temperature after addition of 30 μl lipofectamine. The lipofectamine/DNA/protein mixture was left on the B82 cells for three hours. Twelve hours after the transfection, the infection was carried out with FPV. The cells were processed eight hours after the infection and the CAT reaction performed.

The CAT activity of the transfection control pSV2CAT shows an equally high activity after incubation with increasing protein amounts as without protein (cf. Fig. 3-3). The CAT signal strength with pHL1122 lies, however, as with pHL926 after transfection, at the detection limit, no increase in the CAT activity can be detected in the preparations after addition of

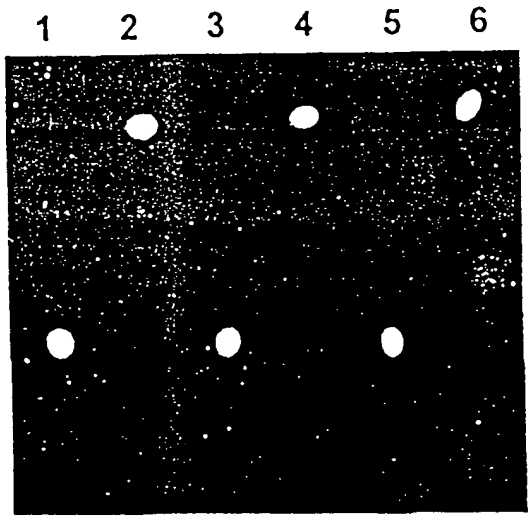


Fig. 3-3: CAT analysis to verify whether the deficiency of the RNA-polymerase I transcriptions system can be improved by "nucleolus targeting"
For the transfection of 10^7 B82 cells, 5 μg plasmid DNA was used in each case. Lane 1: Transfection of pHL926; Lane 2: pSV2CAT; Lane 3: Cotransfection of pHL1122 with 2 μg protein; Lane 4: Cotransfection of pSV2CAT with 2 μg protein; Lane 5: Cotransfection of pHL1122 with 5 μg protein; Lane 6: Cotransfection of pSV2CAT with 5 μg protein.

protein. The control preparation shows that the formation of the liposome vesicles is not negatively affected by the addition of the protein solution. Possibly, the ratio of the DNA quantity to the protein quantity was not optimally selected. Consequently, in deviation from the test performed, the DNA quantities as well as the protein quantities were varied. In all cases, 2.5, 5, and 7.5 μg was incubated with 0.1, 0.5, 1, and 2 μg protein. In this test series as well, the CAT expression could not be increased.

The results show that there was obviously no success in obtaining an increase in expression for the RNA polymerase I system using the "nucleolar targeting" construct. Surprisingly, experiments of G. Neumann yielded the result that the substitution of three nucleotide positions in the promoter region of the viral vRNA results in a significantly increased CAT activity. Consequently, it seemed reasonable to perform the mutation analyses on the basis of these optimized variants.

3.2 The "Promoter-up" Mutants of the vRNA of Segment 4 (HA)

Between the murine rDNA-promoter and the rDNA-terminator, in pHL1169[1], the non-translated end regions of the cDNA (34 bp and 29 bp) of an influenza HA-vRNA are located, flanking the central reading frame in antisense-orientation (cf. Fig. 3-4). Instead of the HA-encoding sequence, for easier in vivo characterization of the mutants constructed at precise points (from AUG to UAA), the reading frame of the reporter gene chloramphenicol acetyltransferase (CAT) was used (Neumann, 1992).

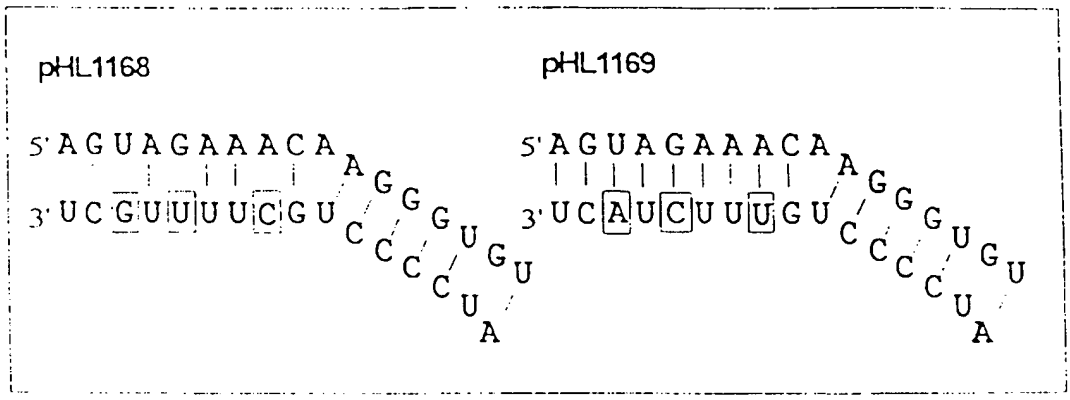


Fig. 3-4: Comparison of the Terminal Sequences of the vRNA of the Segment 4 (HA) in the Constructs pHL1168 and pHL11689
pHL1168 includes the wildtype sequence; pHL1169 includes three nucleotide exchanges on the 3' end of the vRNA (enclosed in a rectangle). (G3'→A3'; U5'→C5'; C8'→U8').. Possible G-U base pairings are depicted as dashed lines; Watson-Crick base pairings are depicted as solid lines.

[1] The plasmids pHL926 and pHL1104 (Neumann & Hobom, 1995) were cleaved for truncation in their vector component with the enzymes *Mlu*I and *Nae*I and the ends filled in with Klenow polymerase. The resultant constructs were named pHL1168 and pHL1169.

Compared to the construct pHL1168, which has the wildtype sequence of the influenza A viruses, in pHL1169, three nucleotide exchanges have been introduced at the 3'-end of the HA nontranslated region in the highly conserved promoter region. Here, the nucleotide positions $\overline{3}$, $\overline{5}$ and $\overline{8}$[2] have been adapted by three substitutions in a complementary manner on the 5' end of the noncoding region. This permits the formation of a complete double strand in the end sections in the vRNA because of the inverted nucleotide sequence of their end regions.

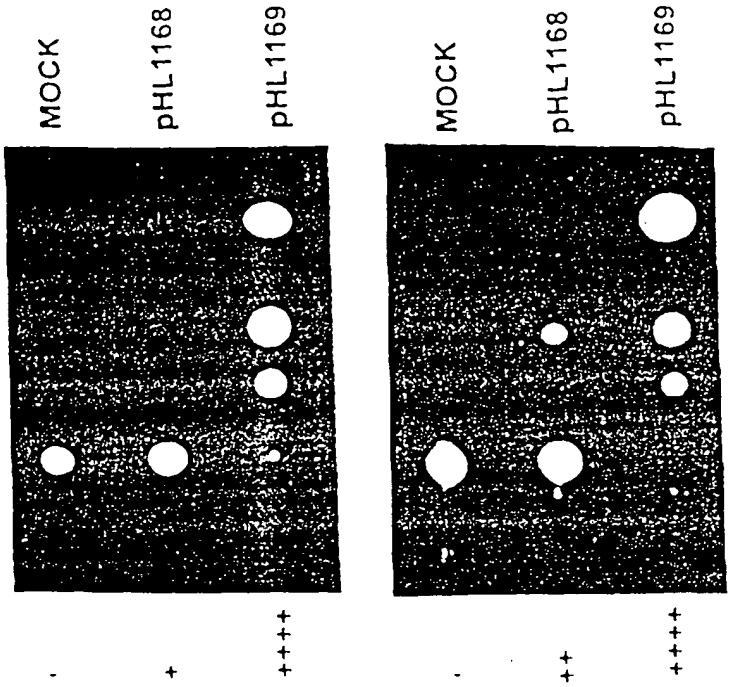


Fig. 3-5: CAT-Analysis of the Constructs That Include a Wildtype vRNA Promoter (pHL1168) and a Promoter with Three Nucleotide Exchanges (pHL1169)
On the left, the results of the CAT-analysis after transfection on B82 cells and subsequent virus infection are shown; on the right, the CAT-activities determined after passage on MDCK-cells are shown.

Evaluation scale for the CAT-reaction:
++++: Reference signal: pHL1169
+++: Highly positive signal
++: Weakly positive signal
+: Very weakly positive signal
-: No signal detectable

[2] Nucleotide positions on the 3' end are noted with an overline.

The advantage of the pHL1169 construct compared to the wildtype situation resides in a significantly intensified promoter activity in the *in vivo* CAT-expression (Neumann & Hobom, 1995). Consequently, starting from the high pHL1169-activity as a reference value, it is possible to detect even weakened promoter variants by means of the CAT-analysis (see Fig. 3-5). Consequently, the promoter variants were constructed in the *in vivo* mutation analysis on this basis and not starting from the less active influenza wildtype sequence, whereby the constructs with optimized promoter structure were included as a standard of comparison and positive controls.

3.3 Generation of Cloning Vectors for the Selective Mutagenesis of the Noncoding Regions

First, it was to be verified whether, in addition to the end sequences for segment 4 RNA molecules with other vRNA-end sequences are also adequate for the amplification and for the packaging of the pseudo-vRNA-molecules.

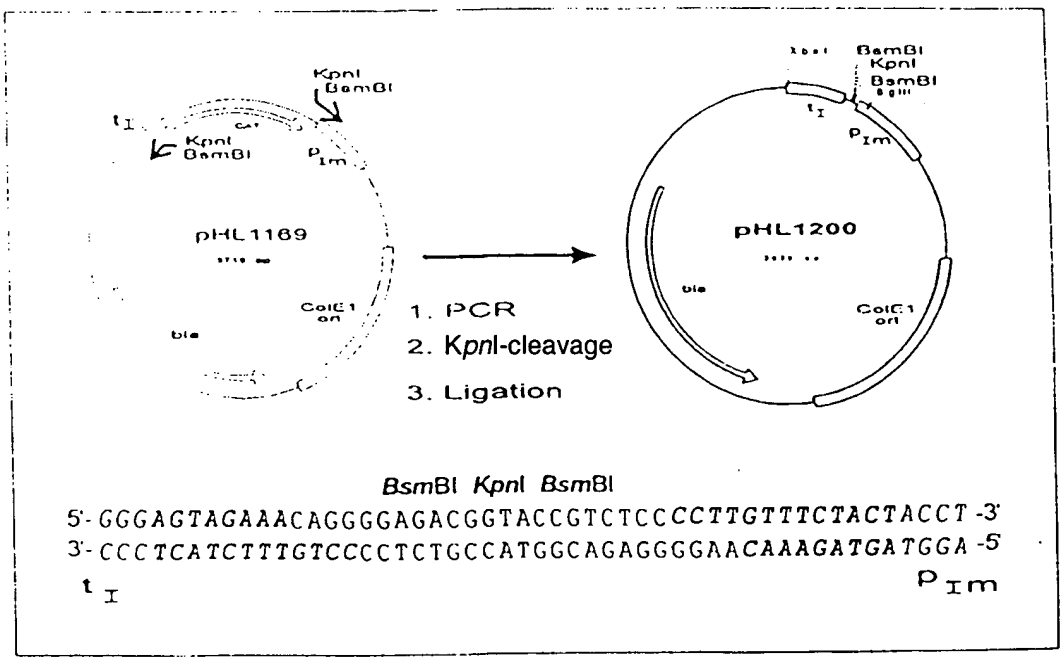


Fig. 3-6: Construction of Universal Vectors for Nucleotidelike Insertion of Arbitrary Fragments in the Noncoding Region

The arrows in the left portion symbolize the primers that are used in a PCR reaction. The oligonucleotides used hybridize with their 3' ends on the plasmid sequence; on the 5' ends they have influenza-specific sequences as well as recognition sequences for the restriction endonucleases *Kpn*I and *Bsm*BI. The fragment resulting from the inverse PCR is cleaved with *Kpn*I. The plasmid obtained after ligation is depicted on the right. The encoding region for influenza-specific end segments is depicted in bold. This sequence is framed by the RNA-polymerase I promoter (p_{Im}) and terminator (t_I).

Since the RNA-polymerase I transcripts must exactly match the influenza ends, it was necessary for the construction of the reporter plasmids that the sequence sections be inserted nucleotidelike between the rDNA promoter and terminator. Furthermore, no additional foreign sequences should be inserted between the nontranslated sequences and the CAT reading frame. It was demonstrated that in the DNA sequences of interest, no suitable restriction cuts were present that would have enabled a nucleotidelike insertion of fragments by direct cloning with restriction endonucleases.

Using the inverse PCR, it is possible to amplify entire plasmids and then to cross-link the blunt ends (Imai *et al.*, 1991). The oligonucleotides used in the PCR for this consist of two parts: The section placed on the 3' end contains the hybridizing sequence necessary for the amplification; the 5' region consists of the altered sequences. A linear DNA molecule was amplified by multiple PCR cycles. After phosphorylation and ligation, the altered DNA sequence was introduced in the region of the fusion boundary. And finally, by sequencing, it was ensured that the sequence actually present matched that desired.

This method was first used to develop constructs that include the nontranslated region of segment 7. Using an oligonucleotide (#PolI-Pan[3]) that hybridizes with the DNA sequence on the 5' end of the viral cDNA and a primer (#M45'vRNA[4]) that hybridizes on the 3' end with the CAT sequence and includes on the 5' end the viral cDNA sequence of segment 7, an inverse PCR was performed (see 2.1.13). pHL1169 was used as a template. The sequence of the resultant constructs was verified by Sanger sequencing to the *NcoI*/*Pvu*II-cuts in the CAT region. The internal *NcoI*/*Pvu*II-section of 401 bp was then replaced by the *NcoI*/*Pvu*II-fragment from pHL1169. The resultant construct was named pHL1286.

[3] #PolI-Pan: 5' – CCTTGTTTCTACTACCTATC-3'

[4] #M45'vRNA: 5'-TAGTTTTTACGCCCGCCCTGC-3

For the construct with a segment 7-specific 3' end of the vRNA, an inverse PCR was performed (template pHL1169, primer #M_NTR1[5]/#M_NTR3[6] [sic parentheses not closed]. The PCR amplification product was cleaved with *Sna*BI and autoligated following phosphorylation. The sequence of the resultant construct was verified by Sanger sequencing to the *NcoI/Pvu*II-cuts in the CAT region. The internal *NcoI/Pvu*II-section of 401 bp was again replaced by the *NcoI/Pvu*II-fragment from pHL1169. The resultant construct was named pHL1195. After cleavage of the plasmid pHL1286 with *NcoI/Bam*HI, a 738 bp *NcoI/Bam*HI fragment from pHL1195 was inserted in the resultant vector fragment. The resultant construct now has M-specific sequences on both ends and was named pHL1303.

In order to be able to insert both 5' and 3' viral cDNA sequences simultaneously into the RNA-polymerase I transcription system, a new system for nucleotidelike mutagenesis was developed. This system uses the restriction endonucleases of type IIs. These enzymes have the property of cleaving not within their recognition sequence, but near it at a specific distance (Beck & Burtscher, 1993). The process consists of two steps. First, a vector with an RNA-polymerase I transcription unit and two internal recognition sequences for the "bracket-enzyme" *Bsm*BI is constructed (see Fig. 3-6). This enzyme has the recognition sequence $^{5'\text{-CGTCTC-}3'}_{3'\text{GCAGAG-}5'}$. With cleavage at a distance of one and five nucleotides from the target sequence, 5'-overhanging ends of four nucleotides develop: $^{5'\text{-CGTCTCNNNNN-}3'}_{3'\text{GCAGAGNNNNN-}5'}$. These single-stranded overhangs are individual, usually asymmetric sequences. After selection of a suitable oligonucleotide, a PCR fragment that has *Bsm*BI cuts on its ends and forms the same complementary ends after restriction cleavage can be inserted in the similarly *Bsm*BI-cleaved vector (see Fig. 3-7). Since the end sequences of the vector cleaved twice with *Bsm*BI have

[5] #M_NTRI: 5'-AATATACGTAGATATTGAAAGATGGAGAAGAAGAACTC-3'

[6] #M_NTR3: 5'-CTGTTTCTACTCCCCC-3'

no nucleotides complementary to each other, no religations are possible after complete cleavage, a circumstance which reduces the proportion of vector clone transformants; the recognition sequence for *Kpn*I inserted works in the same direction, whereby after additional cleavage by *Kpn*I a possibly incomplete *Bsm*BI double cleavage is compensated; i.e., the additional cleavage by *Kpn*I prevents religation of the vector molecule only unilaterally cleaved with *Bsm*BI.

For the construction of such a "universal vector", an inverse PCR was first performed with the primers #1104t1[7] and #1104p1[8]. The plasmid pHL1169 was used as the template. After isolation of the amplification product, the DNA was cleaved on both ends with KpnI and ligated to the ring via these *Kpn*I-ends. To verify the newly introduced DNA sequences, the region between the rDNA-promoter and terminator was sequenced. The plasmid with the proper sequence was named pHL1200 (cf. Fig. 3-6). From this construct, an *XbaI/Bgl*II-fragment including 253 bp was inserted into a vector pHL1169 (2786 bp) cleaved with the same enzymes. The resultant construct is called pHL1234. This vector has the optimized influenza promoter sequence coupled with the murine RNA-polymerase I transcription system. The vector pHL1307 was produced for the expression of vRNA-segments that have a cytosine at position 4 on the 3' end (e.g., NP). For the inverse PCR, the oligonucleotide #PrEH5[9] was used here instead of #1104p1.

[7] #1104t1: 5'-AATGGTACCGTCTCCCCTGTTTCTACTCC-3'

[8] #1104p1: 5'-AATGGTACCGTCTCCCCTTGTTTCTACTACC-3'

[9] #PrEH5:'-AATGGTACCGTCTCCCCTGTTTCCACTCC-3'

In the following, it is shown, using the example of the construction of the plasmid pHL1290, how CAT constructs with influenza ends were produced using the "*Bsm*BI-vector" pHL1234. A PCR was performed with primers #NP5'vCAT[10] and #NP3'vCAT[11] (with pHL1169 as the template). After cleavage of the PCR-fragment with *Bsm*BI, the product was inserted into the vector pHL1234 cleaved with *Bsm*BI (see Fig. 3-7). The sequence of the resultant construct was verified by Sanger sequencing to the *NcoI*/*Pvu*II-cuts in the CAT region. The

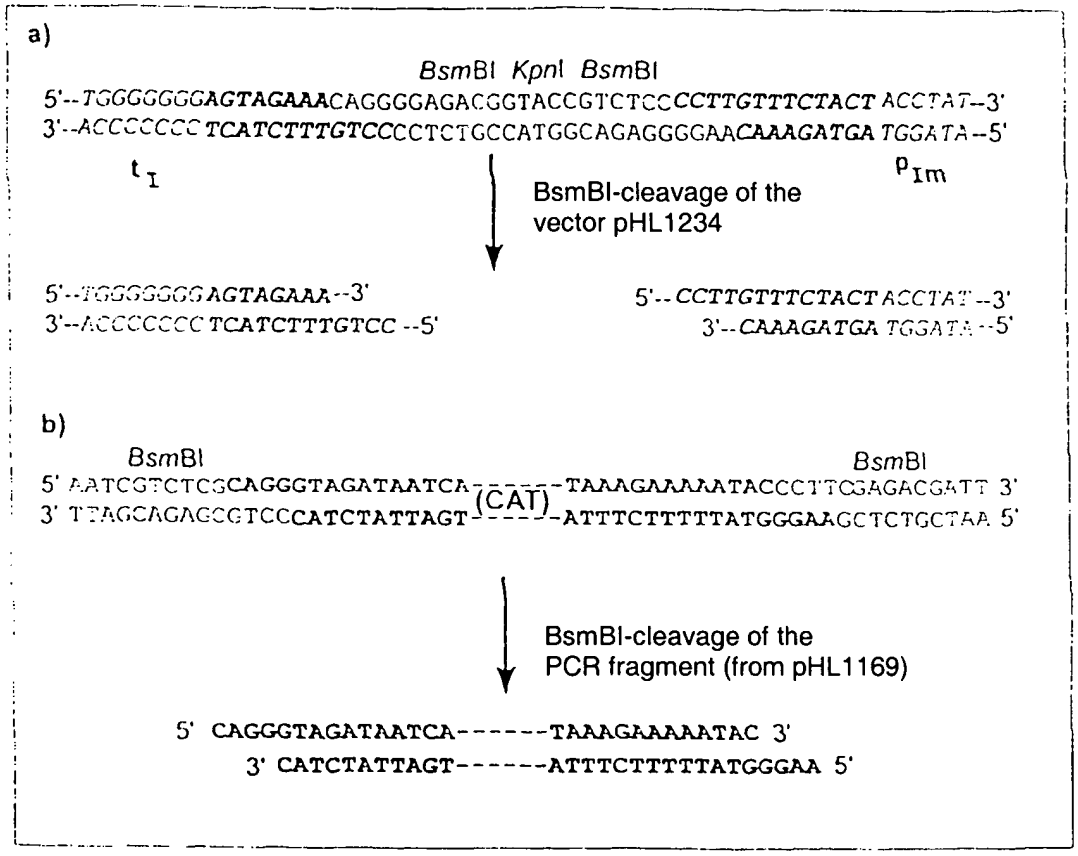


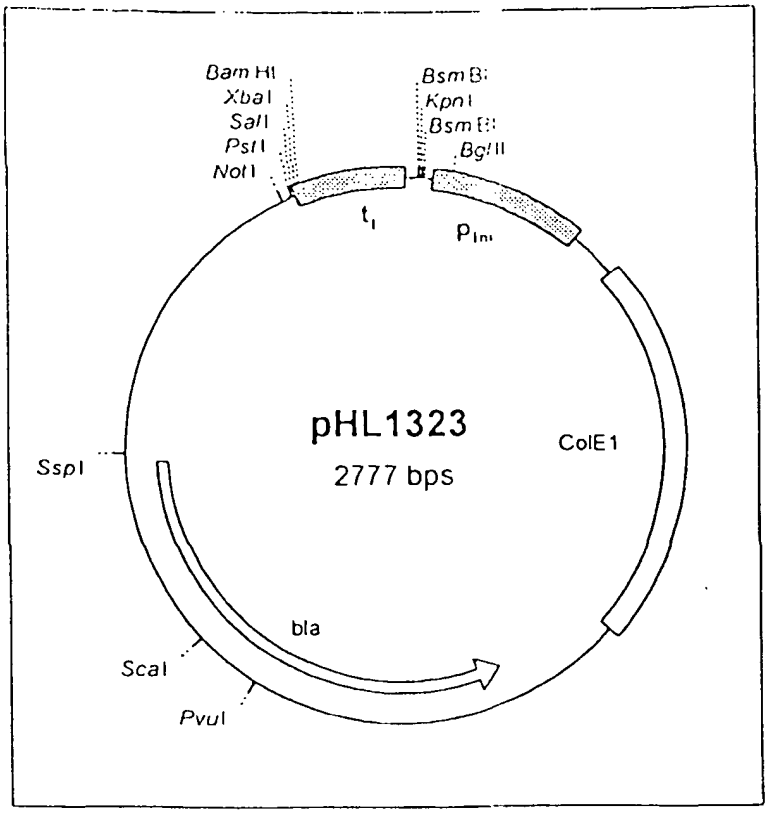
Fig. 3-7: Specific Mutagenesis Through the Use of a Restriction Enzyme of the Type IIs
a) The cleavage of the vector pHL1234 with *Bsm*BI (and with *Kpn*I) results in two single-stranded overhanging ends. The overhanging ends of the cleaved vector fragment are not complementary, a circumstance which prevents vector religation.
b) the PCR amplification product includes on its ends two *Bsm*BI-recognition sequences, arranged such that after cleavage two ends complementary to those in the vector fragment develop.

3 #NP5'vCAT: 5'-AATCGTCTCGAAGGGTATTTTCTTTACGCCCCCGCCTGCCAC-3'

4 #NP3vCAT: 5'-AATCGTCTCGCAGGGTAGATAATCACTCACTGAGTGACATCAAAATCATGGAGAAAAAAACACTG-3'

internal *NcoI/PvuII*-section of 401 bp was again replaced by the *NcoI/PvuII*-fragment from pHL1169. The resultant construct was named pHL1195.

Since cloning using "bracket enzymes" results in a high yield of recombinant plasmid constructs, with pHL1234 the originally present replication origin in the plasmid component was replaced by the "*high copy*" replication origin from pBlueScript II KS, to increase the yield of plasmid-DNA in the processing of bacterial cells. The resultant cloning vector was named pHL1323 (Fig. 3-8). For the nucleotidelike insertion of fragments between the murine rDNA-promoter-sequence and the murine rDNA-terminator-sequence, the vector pHL1324 was constructed. (pHL1324 resulted from the ligation of an *XbaI/BglII*-vector fragment from pHL1323 with the *XbaI/BglII*-fragment including 229 bp from pHL1261 (Flick *et al.*, 1996; Fig. 3-8)).



pHL1323

BsmBI *KpnI* *BsmBI*

5′ -GGGGGA*GTAGAAA*CAGGGGAGACGGTACCGTCTCC*CCTTGTTTCTACTAC*

3′ -CCCCC*TCATCTTTGTCC*CCTCTGCCATGGCAGAGGGGAA*CAAAGATGATG*

t_I C p_{Im}

pHL1324

BsmBI *KpnI* *BsmBI*

5′ -GGGGGGGAGGAGACGGTACCGTCTCTACCTATCTCCAGGTCCAATAGGAC

3′ -CCCCCCCTCCTCTGCCATGGCAGAGATGGATAGAGGTCCAGGTTATCCTG

t_I p_{Im}

Fig. 3-8: The cloning vectors pHL1323 and pHL1324

The restriction map of pHL1323 is presented at the top. At the bottom, the sequences between a murine rDNA-promoter (p_{Im}) and rDNA-terminator (t_I) are shown. pHL1323 contains the constant influenza-specific end sequences (bold) with optimized promoter structure (three underlined nucleotides). After cleavage with *Bsm*BI, the insertion of any influenza cDNA-fragments is possible here, beginning with their segment-specific internal sequences. pHL1324 is identical in the vector portion of the plasmid to pHL1323, but has the *Bsm*BI-cutting sites shifted to the outer limits, such that after *Bsm*BI-cleavage, the nucleotidelike insertion of fragments between rDNA promoter and terminator is possible.

3.4 Detection of the Expression of vRNA Molecules with PB1-, NP-, and M-Specific Segment Ends

The nontranslated region of the RNA segments can be broken down into three regions. The terminal section is identical in all segments (12 nucleotides on the 3' end and 13 nucleotides on the 5' end). Since it was possible to increase the promoter activity by three nucleotides exchanges for segment 4 in this region, the segment-specific sequences of segments 2, 5, and 7 were developed based on this optimized promoter structure (cf. Fig. 3-9). The constant outer portion of the terminal promoter sequence is followed by two to four additional segment-specific nucleotide pairs, which form together a double-stranded element of the promoter structure in extension of the general complementary sequence of three base pairs. Depending on the virus strain and segment, between the terminal elements and the beginning and ending of the reading frame are located 5 to 30 unpaired nucleotides. These contain in the 5' sequence, immediately adjacent, a sequence of six (or five) U-nucleotides, which serve

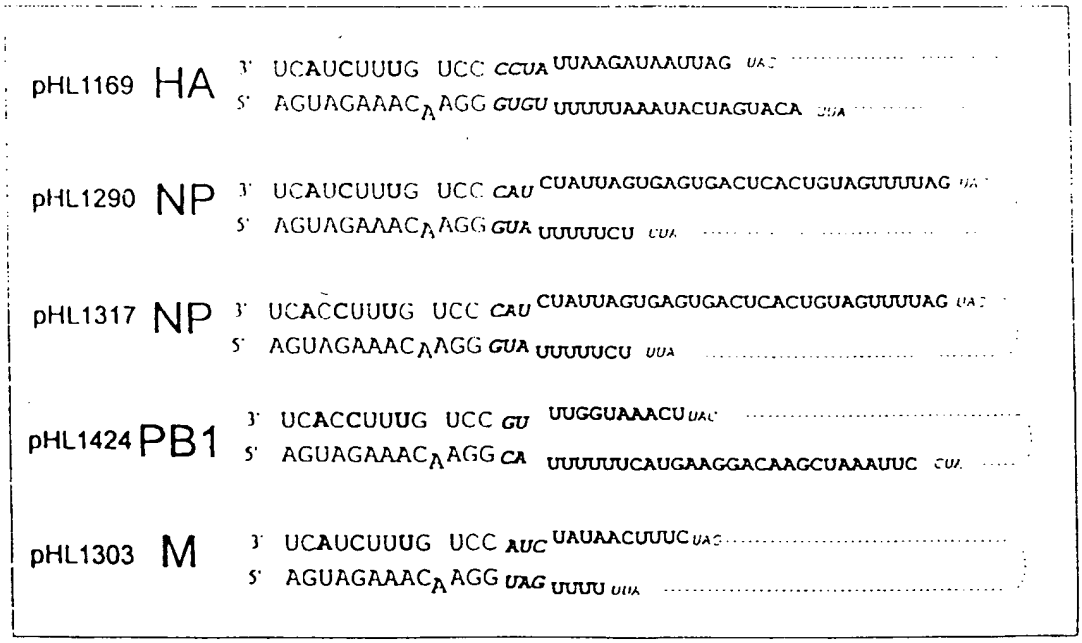


Fig. 3-9: Structure of the Nontranslated End Sequences of Segments 2, 4, 5, and 7
The noncoding section includes, in addition to the highly conserved nucleotides in all eight segments, a segment-specific component of 2 to 4 nucleotide pairs (bold and italics); as well as a section on the 5' and 3' end (bold) of the vRNA varying in length and sequence between the respective segments. According to the constructs listed, the end sequence is presented as an amplified promoter mutant with three nucleotide substitutions (G3A, U5C, C8U). The sequence section encoding for the CAT-protein between the start and stop (complementary UAC and AUU) is symbolized by the dashed line (--)

as polyadenylation templates in the viral mRNA synthesis, but, beyond that, do not permit recognition of any general or specific signal elements.

The constructs for the *in vivo* analysis were produced by means of oligonucleotide-driven mutagenesis with the previously described methods (Fig. 3-9). The segment 2 (pHL1424) has 42 nucleotides on the 5' end and 24 nucleotides on the 3' end (A/FPV). The terminal sequences of segment 7 (pHL1303) include 20 nucleotides on the 5' end and 25 nucleotides on the 3' end (A/PR/8/34). The nontranslated end sequence of the vRNA of segment 5 (pHL1290) includes on the 5' end 23 nucleotides and on the 3' region 45 nucleotides (A/PR/8/34).

After transfection and infection (Section 2.4.6 and 2.4.7), all constructs have an equally high CAT-activity compared to the reference control with HA-specific end sequences (pHL1169) (Fig. 3-10). Also after passage, high CAT-activity signals are detectable with all constructs.

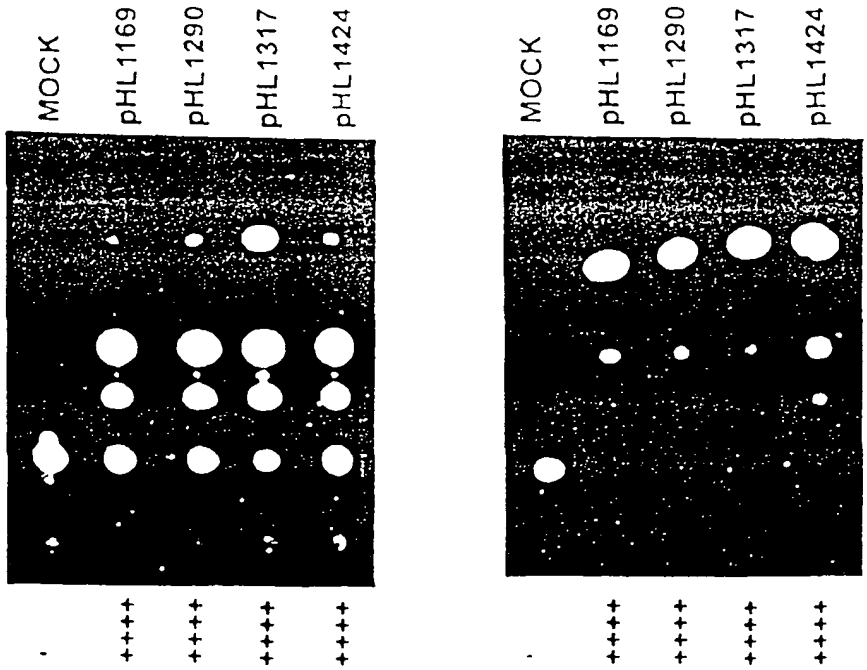


Fig. 3-10: CAT Analysis for Investigation of the Significance of the Terminal Sequences of Segment 2, 5, and 7 for the Replication, Transcripti n, and Packaging of th vRNA
On the left, the CAT-activities after transfection on B82 cells are shown; on the right, the CAT-reactions after the first passage on MDCK-cells.

The noncoding region in the plasmid pHL1290 contains an adenosine residue at position 4 on the 3' end of the vRNA. This corresponds for this position to the promoter sequence of the HA-ends. In contrast, in the wildtype situation in segment 5 at this position, there is a guanosine residue. In the CAT-reaction performed, both NP-variants (4G as well as 4A) have an equally strong signal both after primary infection and also after passage.

The CAT-activity detectable both after transfection/infection and passage makes it clear that all necessary signal structures for the transcription, replication, and packaging are arranged in the noncoding end sequences of the viral RNA molecule.

3.5 Commendations of Segment-Specific Terminal 5' and 3' Sequences of the vRNA

It was concluded from *in vitro* investigations that, for the mRNA-synthesis, the binding of the polymerase complex on only the 3' end of the vRNA is necessary. Whether 5' terminal ends also have an influence on the level of protein expression and thus indirectly on the mRNA-quantity can be tested using the RNA polymerase I expression system *in vivo*. Structural changes in this region are, among other things, possible through the combination of various 5' and 3' segment-specific end sections. If these were to have an influence on the replication or transcription, this would result in a different CAT activity.

For the development of the constructs of interest, the plasmids with 5' and 3' segment-specific sequences within the RNA-polymerase I transcription unit were used. Thus, the plasmids (pHL1169, pHL1290, pHL1303, pHL1424) with cDNA ends of the segments 2, 4, 5, and 7 were used to develop constructs with hybrid terminal sequences by means of a *NcoI*/*Bam*HI cloning step.

3.5.1 Combination of the 5' and 3' Noncoding Regions of RNA Segment 4 (HA) and Segment 5 (NP)

With comparative consideration of various vRNA promoter structures, it becomes clear that the segment-specific nucleotide positions in segment 5 (pHL1290, cf. Figure 3-11) 14 to 16 and $\overline{13}$ through $\overline{15}$ can form a double strand. In contrast to this, in segment 4 (pHL1169), of the four nucleotides each in this region (14 to 17, $\overline{13}$ through $\overline{15}$), only three are present in base pairs. In pHL1298 with 5'-HA and 3'-NP sequences, the nucleotides 14 to 17 and a $\overline{13}$ through $\overline{16}$ have, as in pHL1290, complementary bases at three positions. In the construct pHL1318, the combination of the segment ends permits an additional three base pairings. However, the two variants have sequence differences compared to the authentic structures of segments 4 and 5 or the constructs pHL1169 and pHL1290.

After transfection and subsequent infection, the construct pHL1298, which has on the 5' end an HA-typical and on the 3' and an NP-specific sequence, presents a high CAT activity, which is at the same level as the reference control pHL1169. A slightly weakened CAT-signal is visible with the construct pHL1318 with 3'-HA and 5'-NP sequences. After the passage of the cell culture supernatant on MDCK-cells and subsequent CAT-reaction, both

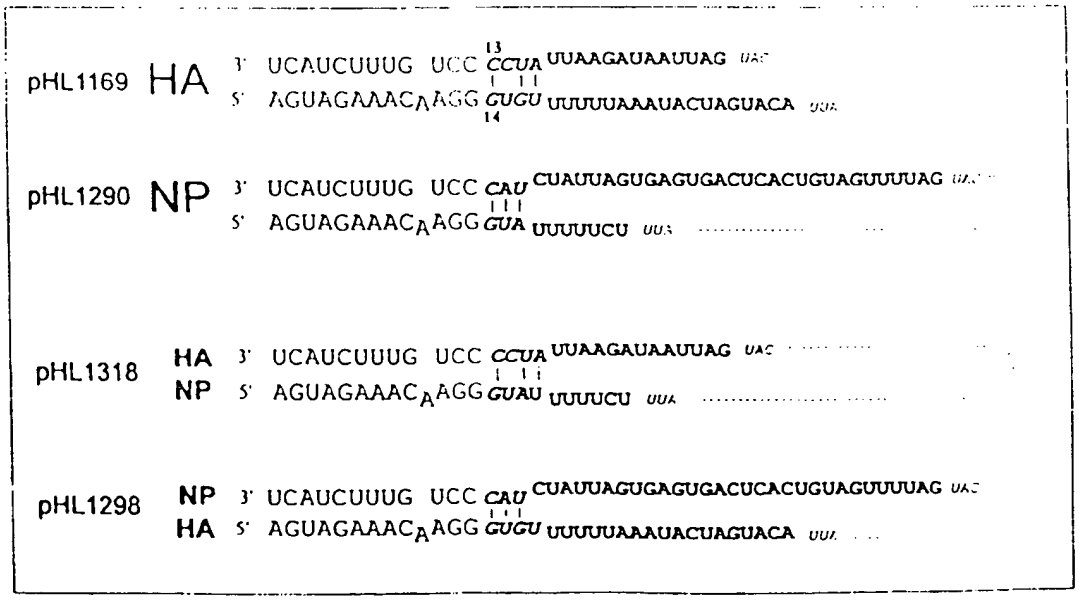


Fig. 3-11: Structure of the Combination Constructs with HA- and NP-Specific End Sequences

combination constructs present a signal strength comparable to the positive control (Fig. 3-12).

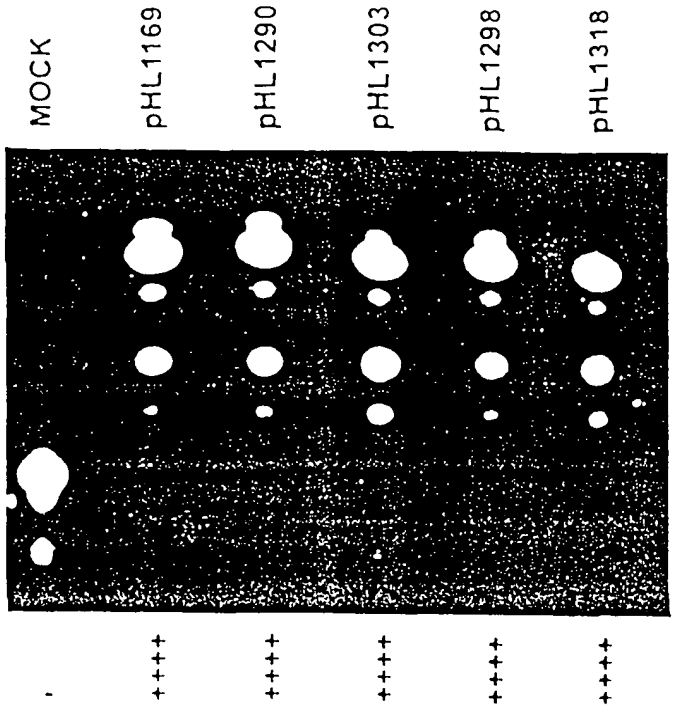


Fig. 3-12: CAT Analysis of the Combination-Constructs with Hybrid vRNA-Ends of Segment 4 and 5 after Secondary Infection of MDCK-cells

3.5.2 Combination of M-Specific Ends with NP- and HA-Sequences

To examine how the combined end structures of segments 4, 5, and 7 affect CAT activity, the constructs pHL1302, pHL1321, pHL1299, and pHL1306 were produced (cf. Fig. 3-13).

The combination constructs pHL1302 and pHL1321 with M- and NP-typical terminal sequences have, despite the lack of base pairing in the region investigated (i.e., in addition to the three base pairs of the conserved sequence), CAT activity both in the primary infection and also after passage on MDCK cells (see Fig. 3-14). A clearly weakened CAT signal is, in contrast, noted with the combinations between HA- and M-ends with likewise missing base pairing potential. From this, it is possible to conclude that, in addition to the extended base pairing, other elements in the 3' ends as well as the 5' ends of the vRNA segments can have an effect on the total RNA-synthesis.

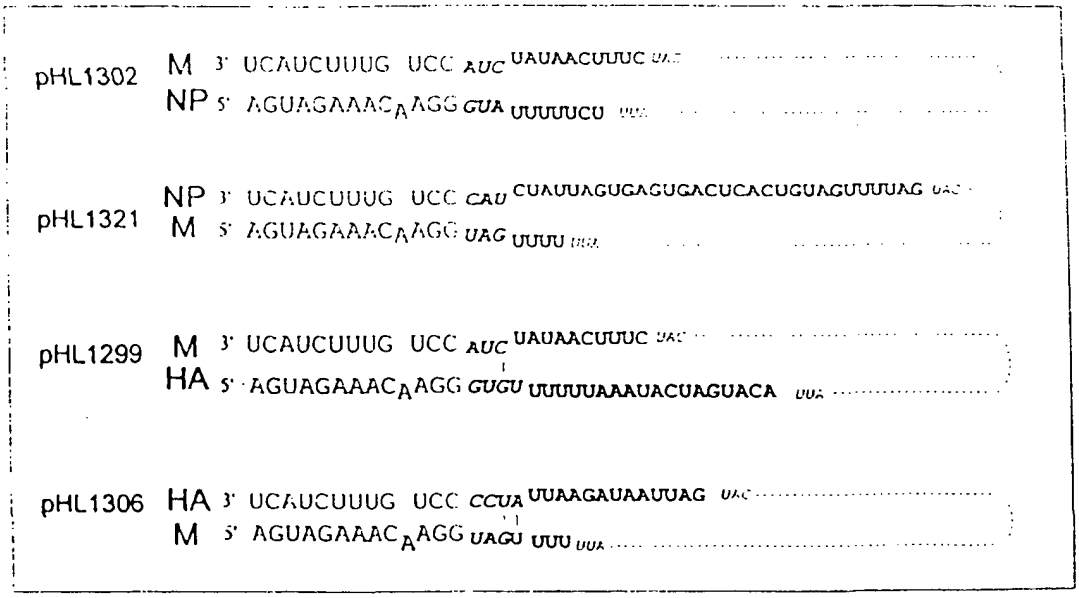


Fig. 3-13: Structure of Constructs with vRNA-Ends of Segment 4, 5, and 7

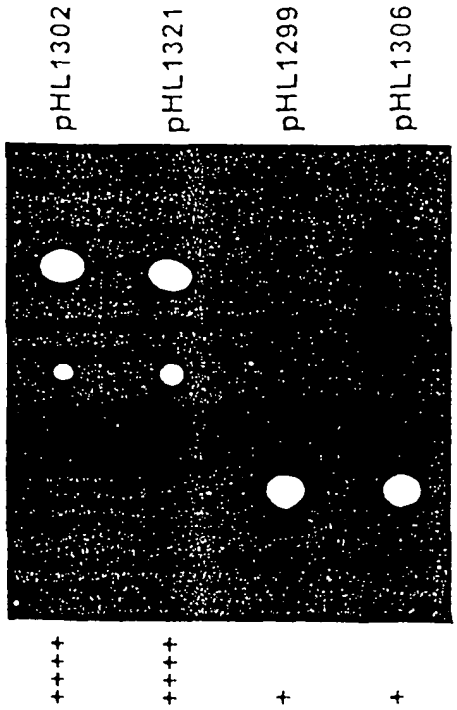


Fig. 3-14: CAT Reaction of the Combination Constructs pHL1302, pHL1321, pHL1299, and pHL1306 after Secondary Infection of MDCK

3.5.3 Combination of PB1-Typical With HA-, NP-, and M-Specific Segment Ends

For further investigation as to whether a CAT activity change can be detected after introduction of a vRNA with PB1-typical end regions, the ends of PB1 were alternatively combined with the M-, HA-, and NP-specific ends (see Fig. 3-15).

The construct pHL145 with PB1-sequences on the 3' end and M-specific 5'-terminal sequences showed a high CAT-activity level both after primary infection and after passage on MDCK cells (Fig. 3-16). The joining of 3'-M-ends with 5'-PB1-sequences in pHL1301 results, in contrast, in no detectable CAT expression. In the construct pHL1297 with 3'-PB1

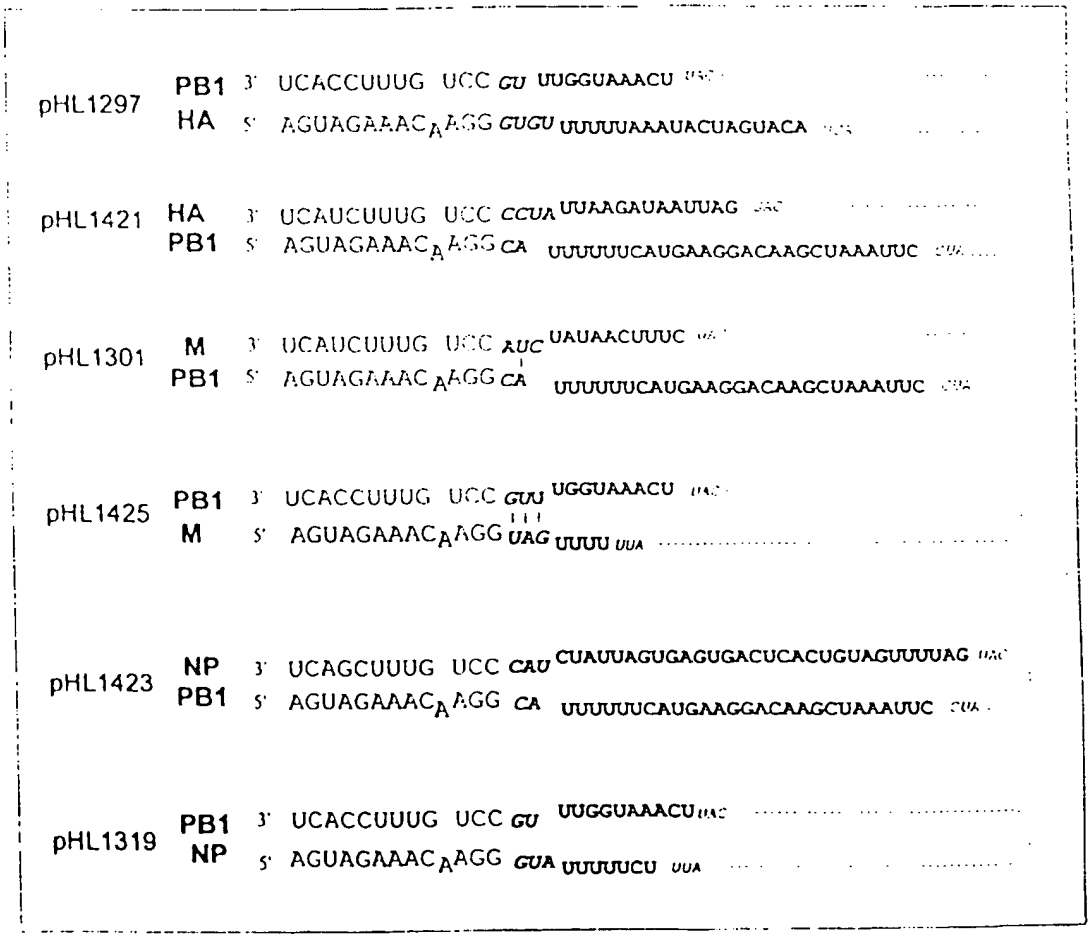


FIG. 3-15: The terminal secti ns of th constructs in which the 5' and 3' vRNA ends of HA, M, and NP were combined with corr sponding nds f PB1

sequences and HA-specific terminal sections as well, no CAT signal is detectable. In contrast, a slight CAT expression is present with the construct pHL1421 that has PB1-ends on the 5' end and HA-ends on the 3' end. The mutant pHL1423 with 3'-NP- and 5'-PB1 terminal sequence elements delivers a weakened, but still detectable CAT signal. The combination of the 5'-NP and 3'-PB1-ends (pHL1319) results in a loss of CAT activity.

The gradation of CAT activities determined points to the fact that the combination of various vRNA segments with ends of different sequences result [sic] in an altered structure of the vvRNA segments. Since the primary sequence of a 5' end or a 3' end is present unchanged in the chimeric constructs compared to the wildtype situation, an activity change can occur only through a conformation change of the terminal regions. Apparently, the secondary structure that is essential for replication and/or polyadenylation in constructs that have a high CAT activity is only affected to a very small extent. Variants with a slight CAT reaction result

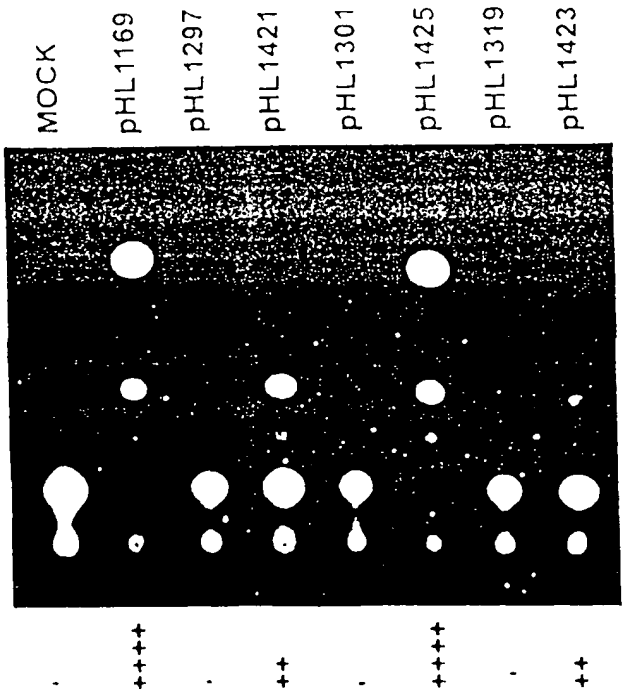


Fig. 3-16: CAT Analysis of pHL1297, pHL1421, pHL1301, pHL1425, pHL1319, and pHL1423

assume an RNA conformation which greatly reduces the synthesis of cRNA as well as the efficiency of the polyadenylation. Negative CAT results area attributable to the loss of the ability to synthesize cRNA and mRNA.

The same expression pattern of the constructs tested after primary infection in B82 cells and after secondary infection in MDCK-cells points to the fact that the packaging of the vRNA segments is changed only insubstantially due to the variation outside the highly conserved region in all eight segments.

The CAT reaction results found support the hypothesis that the 5' end must form a base paired secondary structure with the 3' end of the respective RNA segments that is essential for replication, i.e., cRNA formation and/or polyadenylation.

3.6 Detection of Transcription and Amplification of cRNA

Using the RNA polymerase I expression system, it was first demonstrated by means of *in vitro* transcription that vRNA that has, by definition, negative polarity can be synthesized nucleotidelike with influenza-typical ends (Zobel *et al.*, 1993). The transcription of vRNAs, which results in the formation of mRNAs and then of proteins, and also the subsequent amplification of vRNA via cRNA through the viral polymerase complex with the result of passageable recombinant daughter viruses was likewise detected *in vivo* (Neumann *et al.*, 1994). Additional investigations should now reveal whether it is also possible to form cRNAs

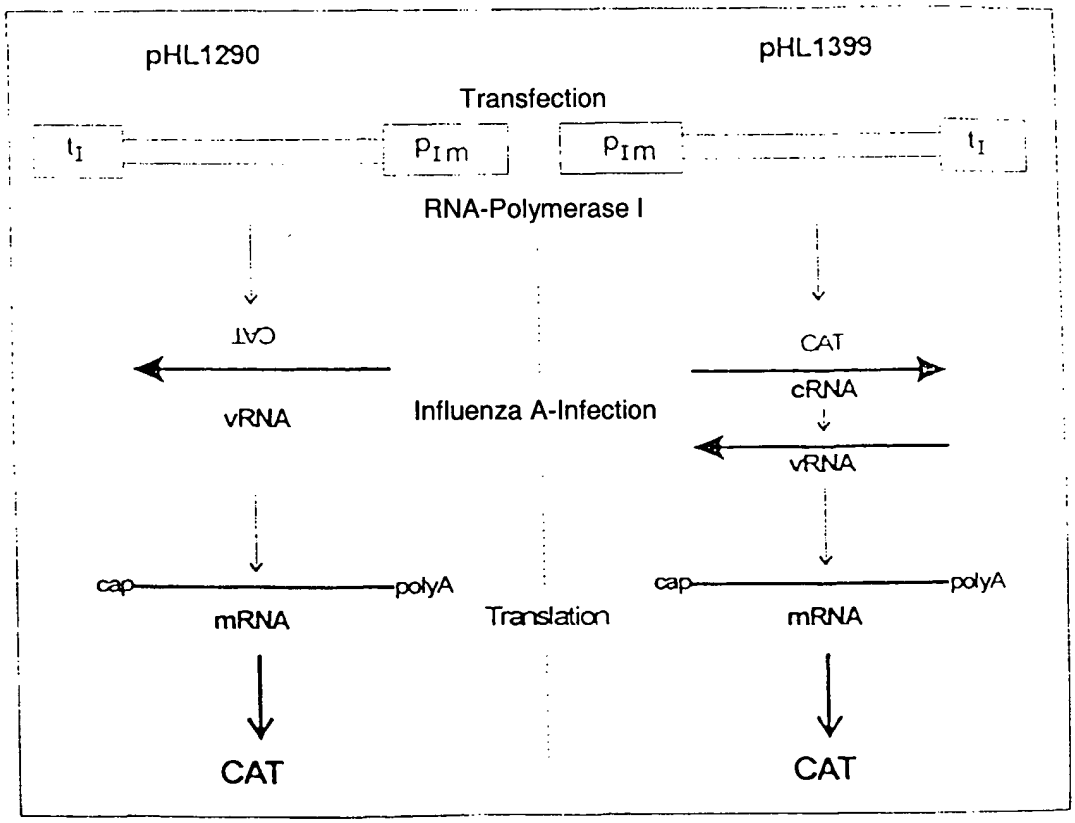


Fig. 3-17: RNA-Polymerase I-Mediated Transcription of vRNA and cRNA
In the constructs pHL1290 and pHL1399, the encoding sequence for the segment 5 was replaced by the reporter gene CAT. This sequence is inserted between the murine polymerase I-promoter (p_{Im}) and the terminator (tI). If the viral polymerase complex recognizes the influenza-specific RNA end sequences after infection, mRNA is synthesized. This is translated into CAT protein and can be detected by means of CAT analysis.

with positive polarity using the RNA polymerase I in the cell, which must then first be copied in the viral replication cycle in vRNA before they can be transcribed in mRNA.

The plasmids pHL1398 and pHL1399 were produced for the development of the constructs with NP-specific segment ends. In comparison with pHL1290, which contains the cDNA in negative polarity between rDNA promoter and terminator, with pHL1399 the CAT sequence was inserted with the influenza ends of positive polarity using the cloning vector pHL1324 (Fig. 3-8) (cf. Fig. 3-17). Analogously to the "promoter-up" mutant with three nucleotide exchanges on the 3' end of the vRNA, with pHL1399 three nucleotides on the 5' end of the cRNA were altered compared to the wildtype (pHL1398) (C3U, A5G, G8A; see Fig. 3-20).

The transfection of the constructs pHL1398 and pHL1399 on B82 cells followed by the superinfection with influenza viruses demonstrated in the CAT reaction with pHL1398

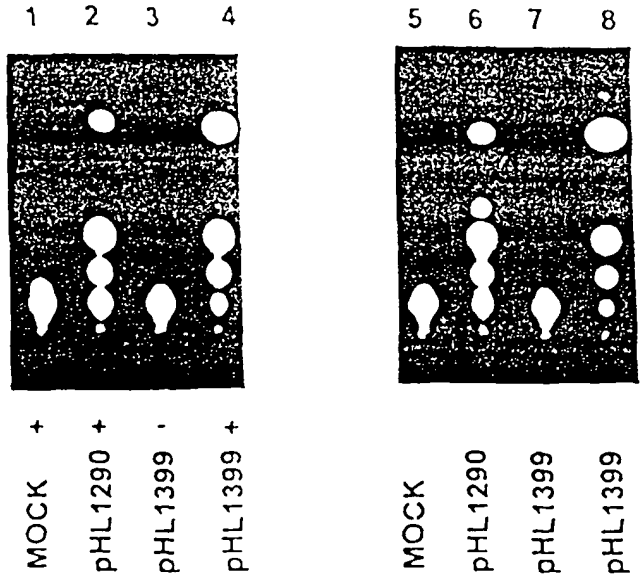


Fig. 3-18: CAT Analysis for the Detection of the Synthesis of Viral mRNA after Primary cRNA Synthesis with RNA Polymerase I.
Lane 1-4: CAT reaction after transfection of the plasmids pHL1290/pHL1399 on B82 cells with (+) and without (-) infection with influenza helper viruses. Lanes 5-8: CAT analysis after passage of the respective supernatants on MDCK cells

low CAT activity (data not shown). In contrast, the construct pHL1399 with the three nucleotide exchanges on the 5' end of the cRNA demonstrated high CAT activity.

Fig. 3-18 shows the results obtained after transfection of the vRNA and cRNA constructs on B82 cells (Lane 1 through Lane 4) and passage on MDCK cells (Lane 5 through Lane 8). Lane 1 records the result of the CAT reaction of mock-transfected, but infected cells. Lane 2 shows the CAT reaction of pHL1290 after transfection and infection. In Lane 3 the CAT reaction of B82 cells that were transfected with pHL1399 but are not infected can be seen. Lane 4 shows the reaction after transfection with pHL1399 and superinfection. Only with subsequent superinfection with helper viruses is CAT activity detectable; in contrast, with transfected but uninfected cells, no CAT expression is detectable. In secondarily infected cells as well, a CAT signal can be discerned (Lane 8).

In both cases, the strength of the CAT activity of the cRNA promoter construct corresponds to that of the reference control pHL1290 with vRNA promoter expression of the CAT mRNA.

To investigate whether there is a temporal difference in the amplification between the two transcripts with different polarity primarily present in the cell, the cells were processed after transfection and infection after different periods of time. The results of the CAT analysis are shown in Fig. 3-19. Four hours after the infection, a slight CAT expression is detectable in both cases. After five hours, CAT activity is clearly detectable both with pHL1290 and with pHL1399. An additional increase in the CAT signal strength is present after six hours with both constructs at the same level at all time points.

The experiments after transfection and primary infection as well as secondary infection made it clear that the cRNA formed by the RNA polymerase I is already transferred in vRNA early in the replication cycle. This genomic RNA is then transcribed in mRNA, a circumstance which results in protein expression. Even with the expression of cRNA, the alteration of the terminal sequence results in an increased promoter efficiency. The three nucleotide

substitutions on the 5' end of the cRNA at positions 3, 5, and 8 correspond here to the standard promoter variations on the 3' end of the vRNA.

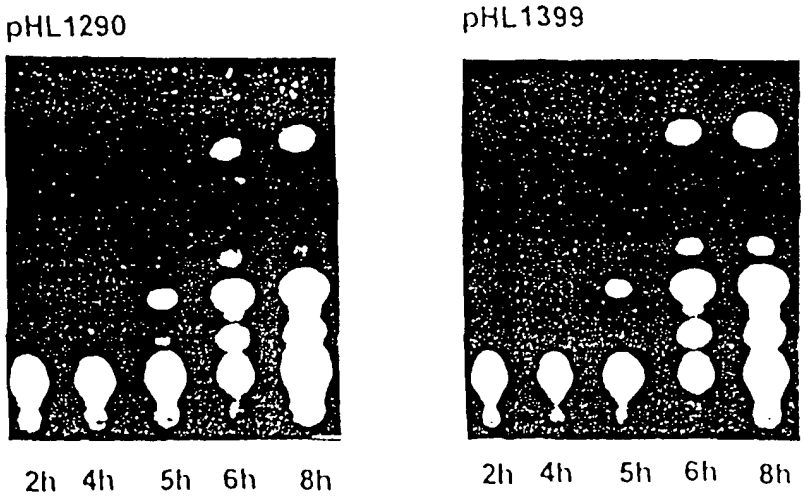


Fig. 3-19: CAT Analysis for Comparison of the mRNA Expression Rates of vRNA and cRNA after Primary Infection.
10^7 B82 cells were transfected with 5 μg DNA. After three hours, the cells were infected with FPV virus (t = 0h). After 2, 4, 6, 8 hours, the cells were processed and the CAT reaction performed.

3.7 Point Mutations in the Proximal Terminal Sequences of the cRNA

The nucleotide substitutions at positions 3 (C→U), 5 (A→G), and 8 (G→A) on the 5' end of the cRNA result in an increased CAT expression in comparison with the wildtype. These nucleotide exchanges now enable three additional base pairings compared to the wildtype: U3-A$\bar{3}$, G5-C$\bar{5}$ and A8-U$\bar{8}$ (Fig. 3-20). The nine terminal nucleotides on the 5' and on the

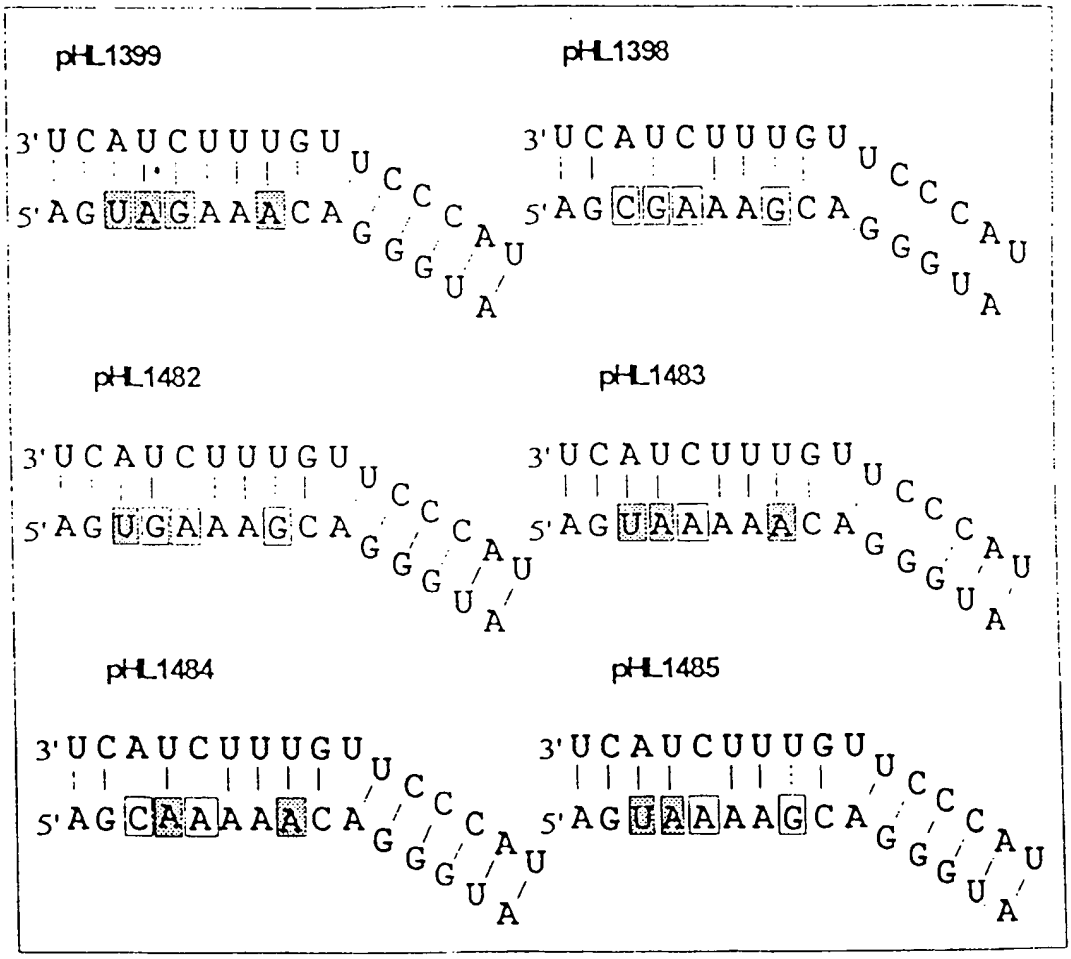


Fig. 3-20: Constructs for the Variation of the Positions 3, 4, 5, 8 of the cRNA Promoter
By nucleotide substitutions, it is examined whether base pairing and/or nucleotide-specific recognition of the polymerase complex in the proximal cRNA promoter element is necessary for the high promoter activity of pHL1399.
The nucleotides that are altered in the construct pHL1399 compared to the wildtype sequence (pHL1398) are highlighted by gray rectangles.

3' end can then form a complete RNA double strand. Additional variations at the nucleotide positions 3, 4, 5, and 8 in this region should now show whether an RNA double strand alone is necessary for the high promoter activity or whether nucleotide substitutions that enable only some of the base pairings also result in higher CAT activity.

For the variation of the nucleotide positions of interest, the corresponding plasmids constructs characterized by oligonucleotide-driven mutagenesis were developed. In the PCR reaction, besides the oligonucleotide #PrEH10[12], the primer #PrEH11[13] was used. After control sequencing, the constructs (pHL1482, pHL1483, pHL1484, pHL1485) schematically depicted in Fig. 3-20 were obtained.

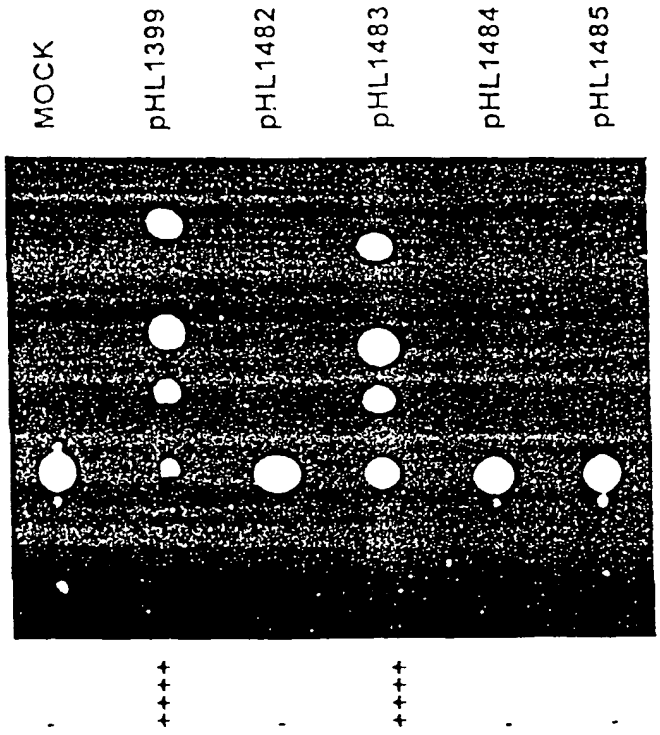


Fig. 3-21: CAT Analysis for the Characterization of the cRNA Promoter

[12] #PrEH10: 5'-AATAGGTACCGAAGACTAGGGAGTAGAAACAAGG-3'

[13] #PrEH11: 5'-AATAGAGCTCGAAGACGGAGGTAG(T/C)(A/G)AAA(A/G:CAGG-3'

After transfection of B82 cells and subsequent influenza infection, the construct pHL1483, which has an adenosine residue on the 5' end of the cRNA compared to the reference control pHL1399 at position 5, demonstrated an equally high CAT signal strength (cf. Fig. 3-21). The elimination of the base pairing between the nucleotides at position 5 on the 5' and on the 3' end (G5C$\bar{5}$ is replaced by A5C$\bar{5}$) results in detectable promoter activity. The conversion of the adenosine residue into a guanosine at position 8 (pHL1485) should enable yet another base pairing with an opposing uridine residue on the 3' end. However, in the CAT reaction, this construct delivers no detectable activity. The mutant pHL1484, which has nucleotide substitutions at position 3 (U→C) and at position 5 (G→A) on the 5' end, is not detectable in the CAT analysis. The triple substitution in pHL1482 (A4→G4, G5→A5, A8→G) likewise results in a complete loss of CAT activity.

In the secondary infection as well, only the constructs pHL1399 and pHL143 show high CAT activity; whereas with the other variants, no CAT signal is detectable. These results clearly show that the formation of an RNA double strand is not sufficient alone for the high promoter efficiency compared to the wildtype.

3.8 Mutagenesis in the Angle Region of the cRNA End Sequences

The cRNA promoter structure can be subdivided into three sections. The nine terminal nucleotides in each case could form an RNA double strand (proximal element). The nucleotides 10 to 15 on the 5' end could form a double-stranded segment (distal element), by base pairing with the nucleotides $\overline{11}$ to $\overline{16}$ on the 3' end. The uridine residue at position $\overline{10}$ on the 3' end of the cRNA must then be present as an additional nucleotide without base pairing. Because of this extra nucleotide, an angle develops in the double-stranded RNA region, which possibly, like the "extra" A10 nucleotide of the vRNA, has significance in the recognition of the cRNA promoter structure because of the viral polymerase complex. Consequently, it was of interest to alter the flexibility of this angle region by deletions and insertions and to determine the resultant promoter activity.

By oligonucleotide driven mutagenesis, the construct pHL1450 with an "angle nucleotide" was developed (see Fig. 3-22). The plasmid pHL1513 resulted from the insertion of a *Pvu*II/*Bam*HI fragment including 743 bp from pHL1399 into the vector pHL1450 cleaved

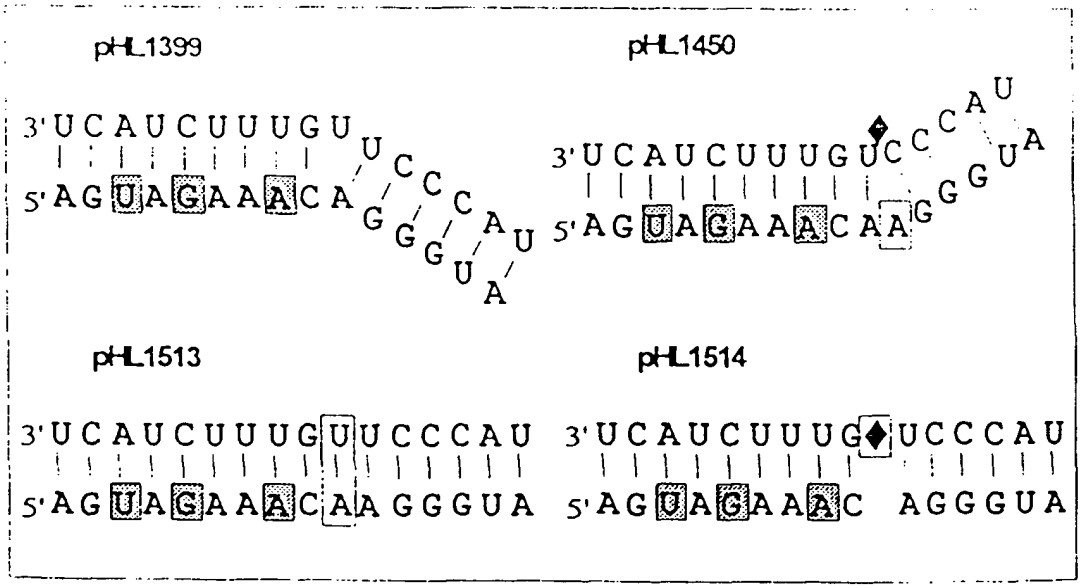


Fig. 3-22: Constructs for the Investigation of the Angle Position of th cRNA Promoter

with *Pvu*II/*Bam*HI. For the development of the plasmid pHL1514, the *Pvu*II/*Bam*HI fragment including 743 bp was isolated from pHL1450. Then, this fragment was ligated into a vector pHL1399 cleaved with *Pvu*II/*Bam*HI.

The deletion of the uridine residue at position $\overline{11}$ on the 3' end of the cRNA and the insertion of an adenosine residue downstream from position 11 on the 5' end (pHL1450) results in a "reversal" of the angle direction between the two promoter elements. After transfection of this construct on B82 cells and subsequent influenza infection, this mutant presents no CAT signal (see Fig. 3-23). The further insertion of a uracil in the opposing position on the 3' end to the inserted adenosine enables a base pairing between these nucleotides. The entire RNA promoter is present with this construct (pHL1513) as a "stretched" RNA double strand. This mutant also delivers no CAT activity in the *in vivo* investigation. The deletion of the uracil residue at position $\overline{10}$ of the 3' end also enables the formation of a stretched, angle-free double strand structure. This construct (pHL1514) also has no detectable CAT activity. In the secondary infection, again, only the positive control pHL1399 presents a signal. The

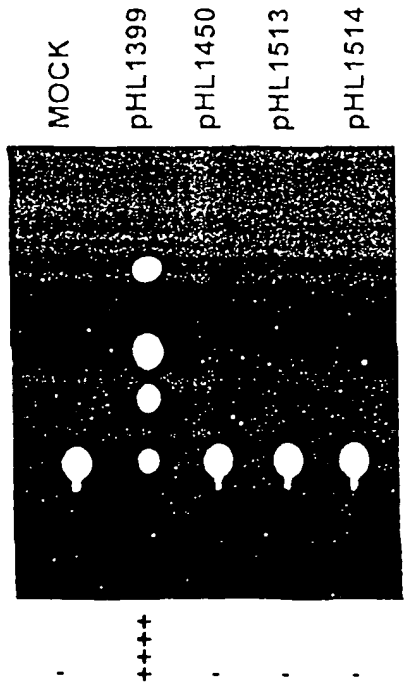


Fig. 3-23: CAT Reaction f r the Investigation f the Angle Region f the cRNA Promoter

described variants in the angle region have no detectable activity.

3.9 Point Mutations in the Distal Terminal Elements of the cRNA

The double strand region of the distal cRNA promoter element is composed of three base pairs ([5'A10G11G12 3']-[3' U$\overline{11}$C$\overline{12}$C$\overline{13}$ 5']), which are the same in all eight segments, followed by segment 5 of three nucleotide pairs of the segment-specific region ([5' G13U14A15 3']-[3' C$\overline{14}$A$\overline{15}$U$\overline{16}$ 5']). Consequently, the double-stranded structure of this section is certainly of significance for the recognition and binding of the viral polymerase. The issue of whether nucleotide-specific recognition is also important for promoter activity can be investigated using individual nucleotide substitutions and compensating double exchanges. This question should be investigated using the example of position 11 on the 5' end and position $\overline{12}$ on the 3' end of the cRNA.

First, the basic constructs (pHL1598, pHL1599, and pHL1600) were constructed by

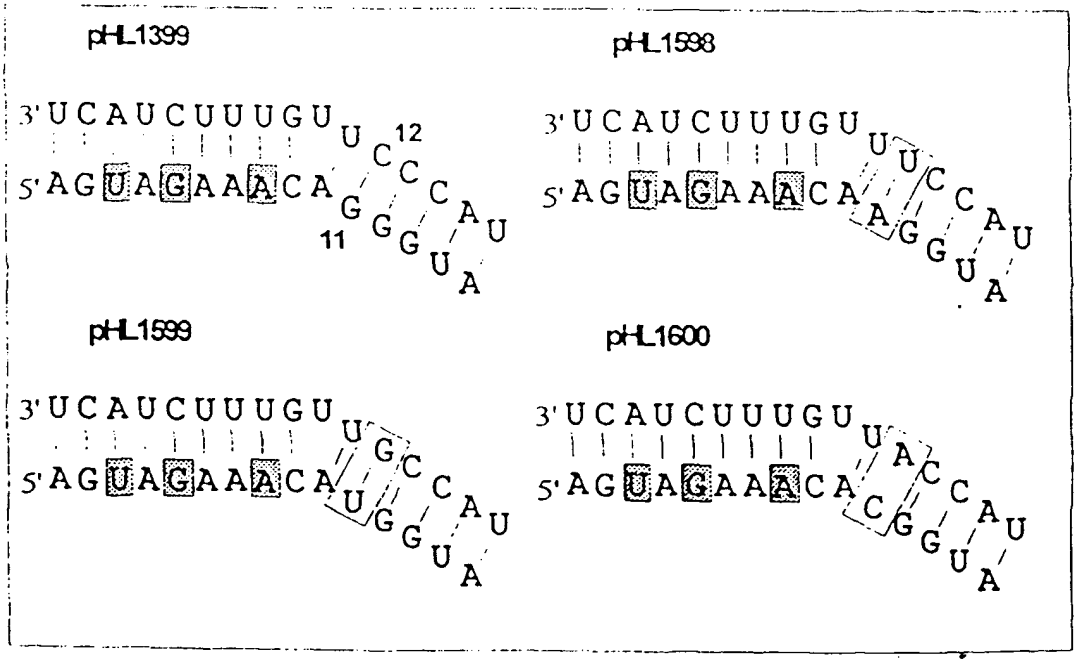


Fig. 3-24: Constructs with P int Mutations at Position 11 on the 5' End and at Position 12 on the 3' End of th cRNA

oligonucleotide-driven mutagenesis: After PCR with the mutation-inducing nucleotides (with pHL1399 as template), the fragment with the open reading frame of the CAT gene and the flanking noncoding sequences was cleaved with *Bbs*I and inserted into the vector pHL1324 cleaved with *Bsm*I (Fig. 3-8). After sequencing and replacement of the original CAT sequence by *NcoI/Pvu*II cloning, the resultant plasmids were named pHL1598, pHL1599, and pHL1600 (see Fig. 3-24). For the combination of constructs, *Eco*RI/*Bam*HI fragments were in each case isolated and ligated together according to the desired nucleotide combinations (pHL1647 through pHL1658, cf. Figure 3-25).

In the CAT analyses performed after transfection and infection in B82 cells, all constructs with individual nucleotide substitutions at position 11 on the 5' end of the cRNA (pHL1650: G11→A11; pHL1653: G11→U11; pHL1656: G11→C11) showed no detectable CAT activity. If, on the 3' end, the cytosine normally present at position 11 is transformed into a uracil (pHL1647), into a guanosine (pHL1648), or into an adenosine (pHL1649), this also results in a loss of the CAT signal. These individual substitutions result in the fact that the nucleotides positioned opposite each other here are no longer present base-paired. By combination of the 5' and 3' ends, it is possible to investigate whether the negative CAT reaction results were caused by a nucleotide-specific recognition of the viral polymerase complex or by the no longer possible base pairing of the nucleotide pairs. The conversion of the cytosine residue $\overline{12}$ from the construct pHL1650 into a uridine residue thus results in an A11-U$\overline{12}$ base pairing in the mutant pHL1598. This compensating double exchange resulted in a strong positive signal in the CAT analysis. The construct pHL1655 with the possible base pair U11-A$\overline{12}$ as well as the mutant pHL1658 with the nucleotide pair C11-G$\overline{12}$ likewise presented a strong positive CAT activity signal. Consequently, all four base pairs are active at this double position. In contrast, for all individual substitutions at the same site, which result in elimination of the base pairing (pHL1600: C11/A$\overline{12}$; pHL1656: C11/C$\overline{12}$; pHL1657: C11/U$\overline{12}$; pHL1651: A11/G$\overline{12}$; pHL1652: A11/A$\overline{12}$; pHL1653: U11/C$\overline{12}$; pHL1654: U11/U$\overline{12}$; pHL1657: C11/U$\overline{12}$), they resulted in no longer detectable

promoter activity. The constructs pHL1599 and pHL1647, in which a G-U base pairing is present in the cRNA promoter structure but an A/C is present in the vRNA promoter sequence, showed no signal in the CAT reaction. In the secondary infection, only the constructs also positive in the primary infection had CAT activity.

The results thus document that the nucleotide pair G11-C$\overline{12}$ of the distal double-stranded section is not recognized nucleotide-specifically. In this section, only the presence of an RNA double strand seems significant for the promoter activity.

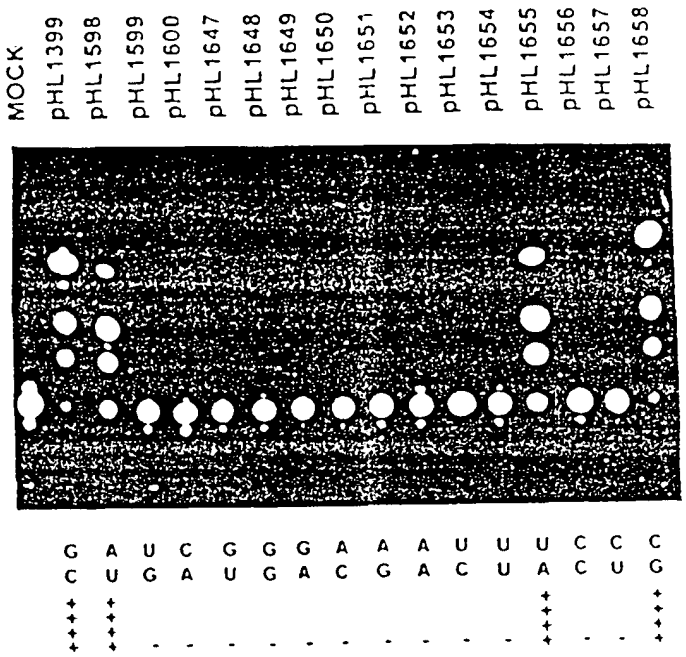


Fig. 3-25: CAT Reaction to the Analysis of the Distal cRNA Promoter Element
By compensating double exchanges, it was investigated whether the distal cRNA segment is recognized as a double-stranded structure or because of nucleotide-specific recognition of the viral polymerase complex. The nucleotides symbolized in the rectangles [sic] represent the nucleotide variants at position 11 on the 5' end (top row) and at position $\overline{12}$ on the 3' end of the cRNA (bottom row).

3.10 Deletion Mutants in the Nontranslated Sequence of Segment 5

In all influenza A virus strains, the nontranslated region of segment 5 includes 46 nucleotides on the 5' end of the cRNA. Whereas the terminal sequences (16 nucleotides on the 5' end; 15 nucleotides on the 3' end) must be present in a defined secondary structure in order to exercise their function via the binding of the polymerase complex, the role of the remaining nucleotides on the 5' end has hardly been investigated. Consequently, it should first be investigated whether deletions in this region result in a change in CAT activity. The thing to do here was to subdivide this section into a portion with a palindromic sequence that could form a "stem loop" structure (position 21 through 35) and the sequences surrounding this region on the left and right.

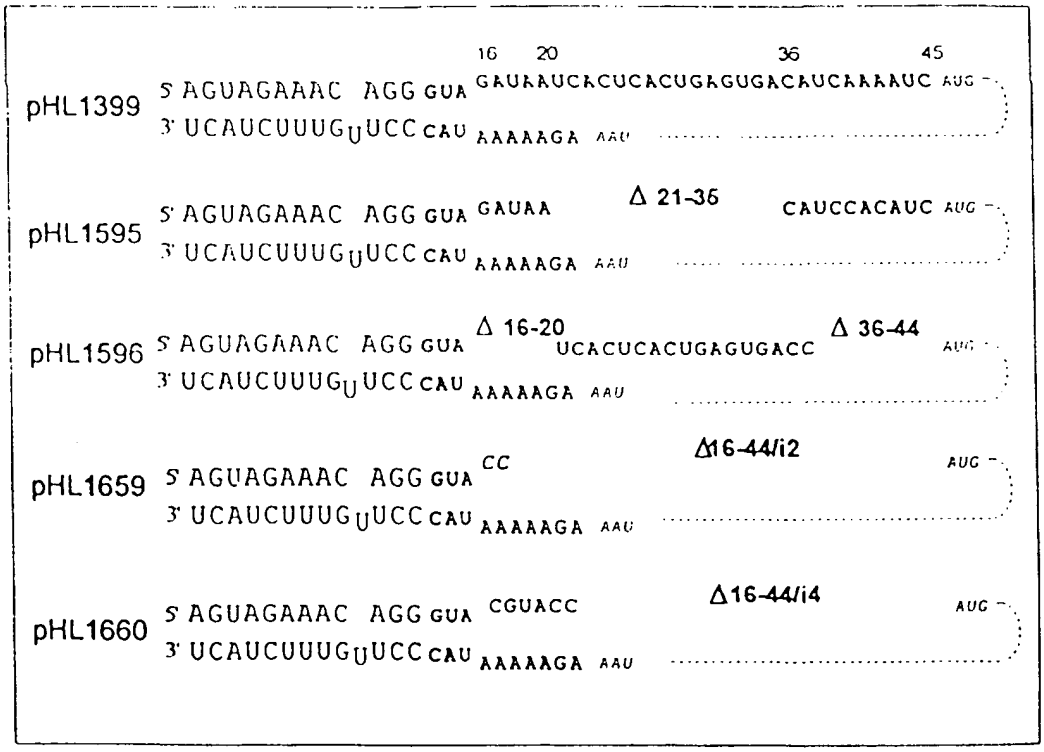


Fig. 3-26: Structure of Constructs with Nucleotide Deletions on the 5' End of the cRNA
The terminal 5' and 3' sequences are schematically depicted as double-stranded. The deleted nucleotide positions are marked by a Δ. The dashed line symbolizes the encoding region for the reporter gene CAT.

Using PCR, DNA fragments were amplified with a CAT reading frame and with the ends altered as desired. The oligonucleotide #PrEH10 was used for the amplification of the 3' end. The oligonucleotides #PrEH22[14] (pHL1659), and #PrEH23[15] (pHL1595) #PrEH24[16] (pHL1596), were used as 5' primers. After cleavage of the PCR products with *Bbs*I and insertion in the vector pHL1324 cleaved with *Bsm*BI (Fig. 3-8), the 3' ends were sequenced to the *Pvu*II cut. The remaining plasmid portion was replaced by a *Pvu*II/*Bam*HI fragment from pHL1399 including 743 bp (pHL1595, pHL1596). With pHL1659 replacement was

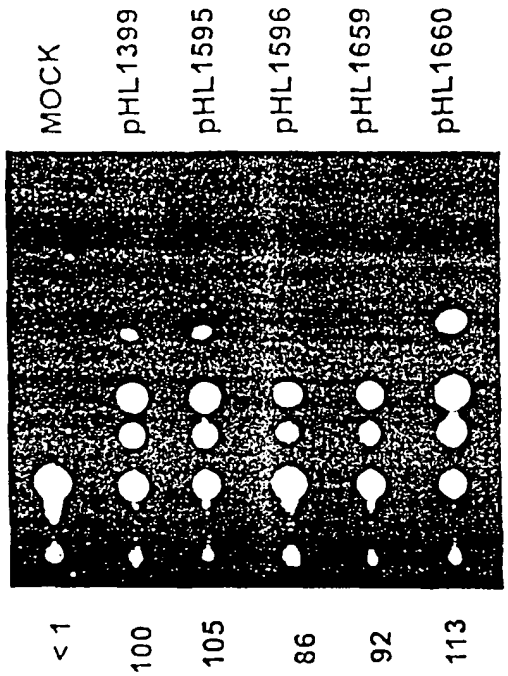


Fig. 3-27: CAT Analysis of Deletion Mutants in the 5' Section of the cRNA
After secondary infection of MDCK cells, the cells were processed and the CAT reactions performed with 50 µl of a 1 : 1000 dilution of the cell lysate. The CAT activity is presented in comparison with the reference control (pHL1399).

[14] #PrEH22: [Illegible]

[15] #PrEH24: [Illegible]

[16] #PrEH23: [Illegible]

performed in the preliminary construct by means of *Kpn*I/*Bam*HI (an 858-bp-long fragment from pHL1558). pHL1660 resulted from pHL1659 after cleavage with *Asp*718 and filling in the 5' overhanging ends with Klenow polymerase (see Fig. 3-26).

After transfection of the constructs on B82 cells and infection, a strong CAT reaction result can be detected in all variants. In the secondarily infected MDCK cells as well, a high CAT activity is detectable. Even after a 1 : 100 dilution of the cell lysate, there was no significant difference in the level of expression in the primarily infected cells; and only after 1 : 1000 dilution of the extracted proteins used from the secondarily infected cells, are very slight signal differences in the CAT enzyme reaction discernible (Fig. 3-27).

3.11 Nucleotide Insertions in the Nontranslated Region of Segment 5

Deletions in the 5' nontranslated region between position 16 and 45 resulted in only a slight impairment of the CAT activity. Consequently, it was of interest to investigate how the level of protein expression is altered by lengthening the terminal section.

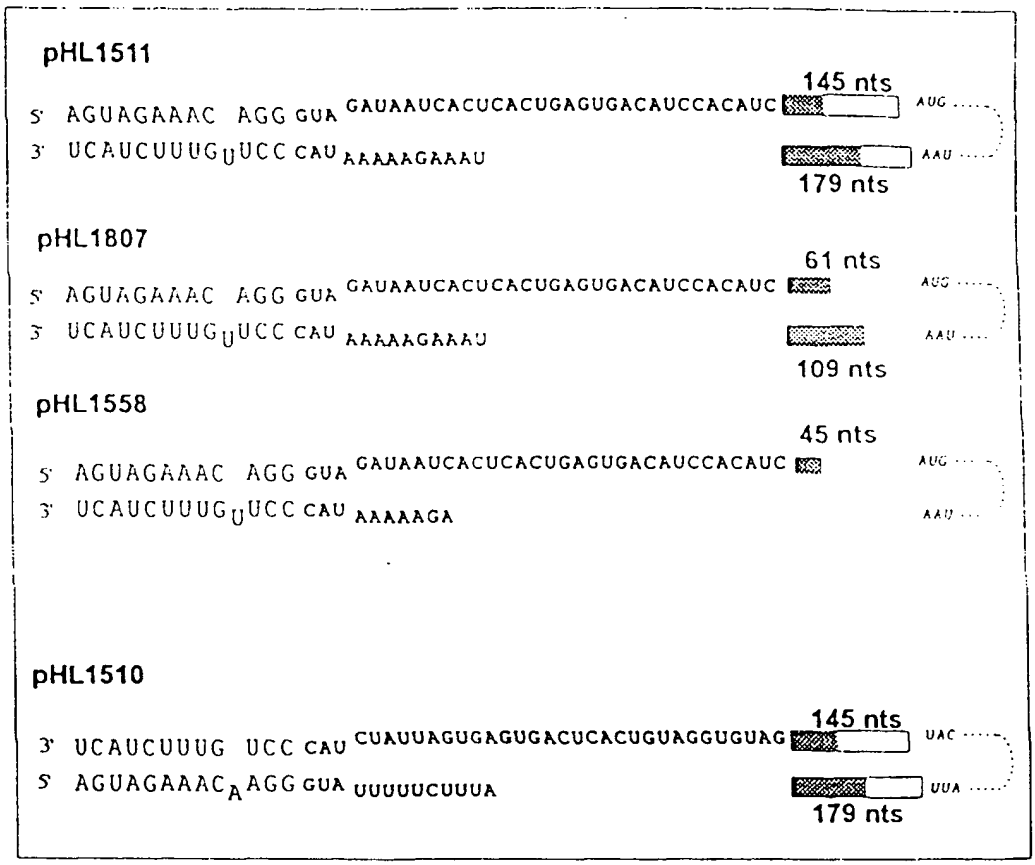


Fig. 3-28: Structure of the Constructs with Inserted Foreign Sequences in the Nontranslated Region of Segment 5
The rectangles symbolize the position of the inserted foreign sequences. The length of the inserted sequences is indicated either above or below the respective rectangles. The gray areas symbolize sequences from the cloning vector pBlueScriptII KS; the white rectangles represent sequences from the plasmid pcDNA3CAT. The RNA sequences presented match the polarity, as present after transcription of the RNA polymerase I.

To develop the insertion constructs, a *Bss*HII cut was first introduced between 5' and 3' end of the noncoding sequence: By means of inverse PCR, using the oligonucleotides #PrEH20[17]/#PrEH21[18] and the plasmids pHL1395 and pHL1472 serving as templates, the linear fragments were cleaved with *Bss*HII. After ligation of the cleavage products, the plasmids pHL1496 and pHL1497 were obtained. A *Bgl*II/*Xba*I fragment with the murine terminator sequence and including 304 bp and with the altered noncoding region was inserted in a 2524-bp-long pHL1323 vector fragment. The resultant constructs were named pHL1499 and pHL1500. After cleavage of these plasmids with *Bss*HII, an 839-bp fragment from pHL1493 was inserted. (pHL1493 originated in the combination of a 2566-bp *Hin*dIII vector fragment from pHL1322 and a 666-bp-long fragment with the reading frame for the reporter gene CAT from pcDNA3CAT.) After characterization of the orientation of the *Bss*HII insertion by restriction cleavage, the "vRNA construct" was named pHL1510; and the "cvRNA construct", pHL1511 (analogous to pHL1290 and pHL1399; see Fig. 3-17). These plasmids contain additional sequences in the nontranslated region that originated in the cloning vectors pBlueScriptII KS (Stratagene) and pcDNA3CAT (Invitrogen). These constructs include before the start of the reading frame an additional 145 nucleotides; after the stop codon, an additional 179 nucleotides had been inserted (see Fig. 3-28). To develop a plasmid with shorter insertions, the vector pHH3 (see 3.13) was cleaved with *Sac*II/*Eco*RI and ligated with a PCR-CAT fragment (with the oligonucleotides used #CAT-InsI[19]/#CAT-InsII[20]) after *Sac*II/*Eco*RI cleavage. After sequencing and replacement of the CAT sequence by a *Pvu*II/*Bsm*BI cloning step, the plasmid pHL1807 was obtained. It contains an additional 61 nucleotides before the start of the CAT reading frame and a foreign fragment including 109 nucleotides from the cloning vector pBlueScriptII KS after the stop codon. A foreign sequence including 45 nucleotides, which originated from the plasmid pBlueScriptII KS, was

[17] #PrEH20: 5'-ACCTGCGCGCTCGCGATGTGGATGTCACTC-3'

[18] #PrEH21: 5'-ACCTGCGCGCAGGCCTAGCTAGGTAAAGAAAAATACC-3'

[19] #CAT-InsI: 5'-AATACCGCGGTCTCCCATGGAGAAGAAAATCACTG-3'

[20] #CAT-InsII: 5'-ATCGAATTCGGTCTCCTTTACGCCCCGCCCTG-3'

introduced in the construct pHL1558. (The plasmid pHL1558 resulted from the ligation of a *Kpn*I/*Bam*HI vector fragment (pHL1512) including 2468 bp and an 858-long *Kpn*I/*Bam*HI fragment from pHL1515).

In the primarily infected B82 cells, the constructs pHL1510, pHL1511, pHL1558, and pHL1807 on B82 cells have high CAT activity. In the secondary infection, the variants that delivered positive CAT signals in the primary infection also have high CAT activity.

After 1 : 100 dilution of the MDCK cell lysate used, the insertion constructs present a slight weakening compared to the CAT signal strength of the reference control pHL1399 (Fig. 3-29).

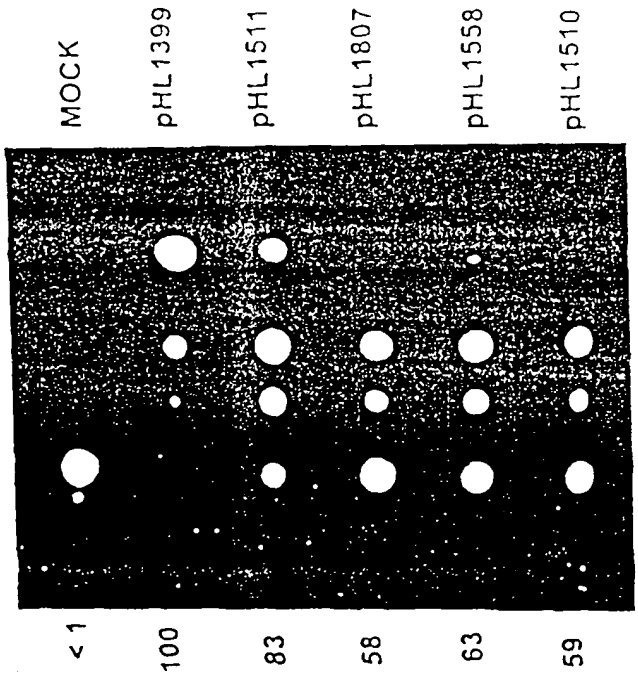


Fig. 3-29: CAT Analysis of the 5' cRNA Insertion Constructs after Dilution
After passage of the virus supernatant from the primary infection on MDCK cells, the cells were processed and the cell lysate used, after dilution (1 : 100), for the CAT reaction.

3.12 Detection of Viral RNA by RT-PCR

The insertion of relatively long foreign sequences with clearly deviating structure into the nontranslated region of segment 5 resulted in virtually unweakened positive signals in the CAT analyses. Further investigations should be performed to determine whether this system is also suitable for the expression of influenza genes proper. The detection of the transcription and packaging of the viral RNA formed can be verified after passage by means of RT-PCR. Since additional sequences are contained in the RNA segment inserted, in comparison with the NP segment already present in the virion, this section can be used for the specific detection of these RNA molecules. Through the selection of primers which can hybridize only on this foreign sequence, only the respective RNA molecules that contain this subregion are amplified in the reverse transcription as well as in the subsequent PCR reaction. This experimental procedure thus enables differentiation between segment 5 of the helper virus and the recombinant segment form after transfection and infection.

For the development of the expression construct, the plasmid pHL1375 was used. It contains the nucleoprotein encoding sequence from the influenza strain A/FPV/Giessen. After the cleavage of pHL1499 with the restriction endonuclease *Bss*HII, a 1644-bp fragment from pHL1375 was inserted in the resultant vector fragment. The desired orientation was verified by restriction cleavage. The resultant construct is named pHL1563 (see Fig. 3-30).

Three hours after transfection of B82 cells with pHL1563-DNA (5 μg/30 μg lipofectamine), the cells were infected with the virus strain FPV/Bratislava. In a parallel preparation, the construct pHL1510, which has a CAT reading frame, was used as a positive control (see Section 3.11). After eight hours, an aliquot of the cell supernatant was passaged on MDCK cells. After 16 hours, MDCK cells were infected with the virus-containing cell supernatant of the primary passage. After 24 hours, all cellular RNA was isolated (see 2.2.1). With the

RNA obtained, a reverse transcription reaction was performed in each case and followed by a PCR reaction (see 2.2.3).

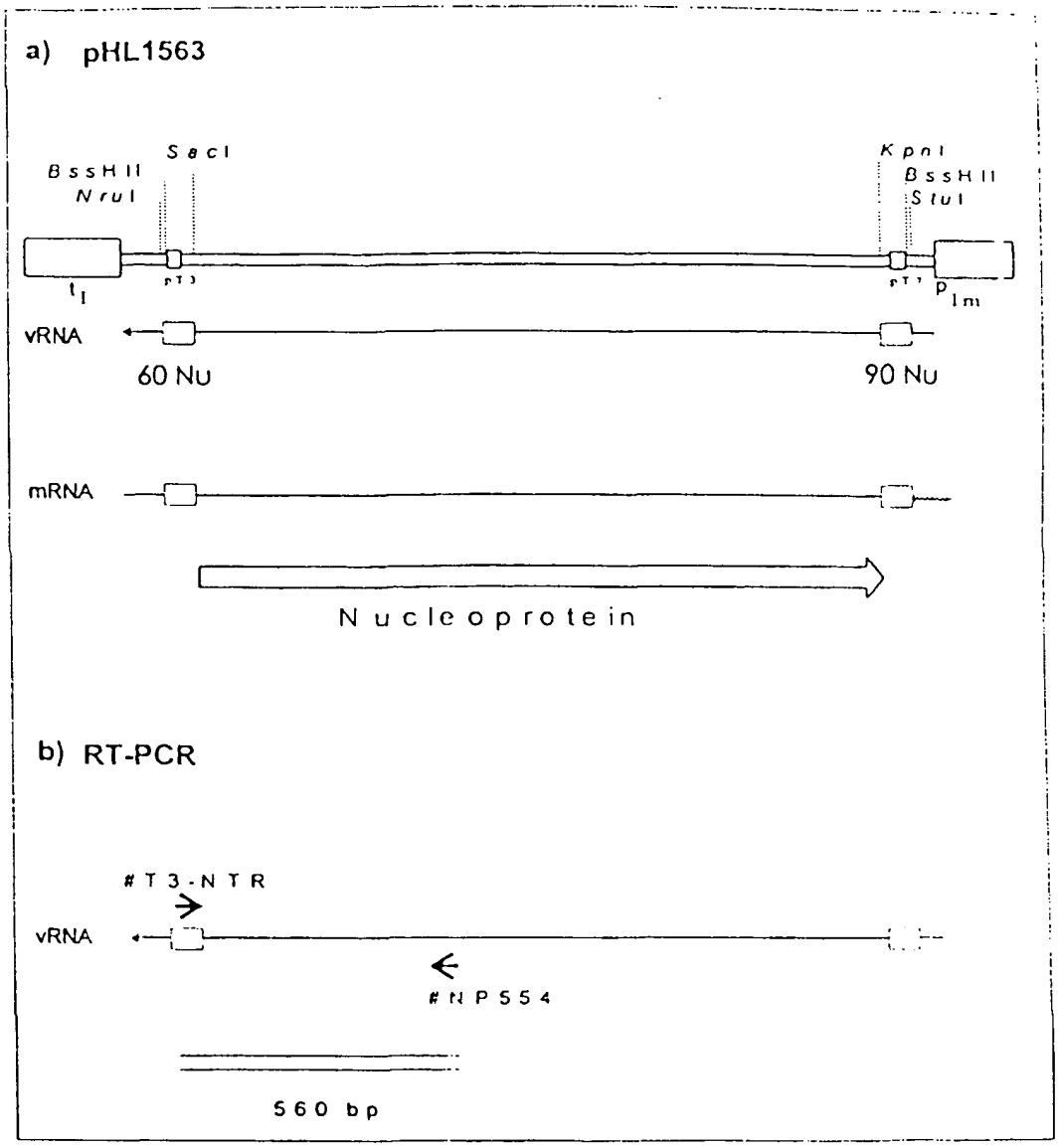


Fig. 3-30: Recombinant Expression of the Nucleoprotein and Detection by Means of RT-PCR
At the top (a), the structure of the construct pHL1563 and the transcription products anticipated after infection are presented. The sequence for the nontranslated region of segment 5 with the enhanced promoter sequence is located between the murine RNA polymerase I promoter and the terminator. The encoding sequence of the nucleoprotein has been inserted between the *Sac*I- and *Kpn*I-recognition sequences. Foreign sequences are introduced before and after the NP reading frame. These inserted sections enable the specific detection of the foreign sequences in the viral RNA molecules depending on the selection of suitable oligonucleotides (symbolized by arrows). (b) The introduction of a genetic marker thus enables the differentiation between helper virus NP and a recombinant NP-RNA molecule.

With pHL1510 and pHL1563, the oligonucleotide #T3-NTR[21] was used in the RT-PCR reaction. In the RT-PCR reactions, with pHL1510, the CAT-specific oligonucleotide #CAT-RTPCR[22] was used; with pHL1563, the primer #NP554[23]. As a control for any possibly occurring plasmid DNA contaminations, a preparation without the addition of RT enzyme was also run. The cyclical PCR reaction ran for 30 seconds at 94°C, 30 seconds at 65°C, and 30 seconds at 72°C, whereby 40 cycles were performed. After electrophoretic separation of an aliquot on an agarose gel, in the detection of the NP-RNA, only a weak band was discernible. Consequently, after addition of *Tfl*-polymerase (5 U), an additional 20 cycles was performed with the remaining preparation.

Fig. 3-31a presents the results obtained after the gel electrophoretic separation for pHL1510. In Lane 2, a DNA fragment of the anticipated size of 413 bp, which results from the PCR amplification with the oligonucleotides #T3-NTR and #CAT-RTPCR, is discernible. No fragment develops when an RT reaction preparation without the addition of reverse transcriptase is used for the PCR reaction (Lane 1). The specificity of the amplification is reflected in the control preparations, in which in the RT-PCR reaction only one of the primers was used in each case (Lane 3 and Lane 4).

The investigation of the RT-PCR reaction for pHL1563 is shown in Fig. 3-31b. In Lane 1, an aliquot of the reaction preparation in which the primers #T3-NTR and #NP554 are used is recorded. With this preparation, a DNA fragment of the anticipated size of 560 bp is discernible. No amplification product develops after the PCR reaction when the no reverse transcriptase was added in the RT reaction (Lane 2) or when only one oligonucleotide was used in each case in the RT-PCR reactions (Lane 3 and Lane 4).

[21] #T3-NTR: 5'-AATTAACCCTCACTAAAGGGAACAAAAG-3'

[22] #CAT-RTPCR: 5'-GTGTAACAAGGGTGAACACTATCCC-3'

[23] #NP554: 5'-GAACCTTGCATCAGAGAGCACAT-3'

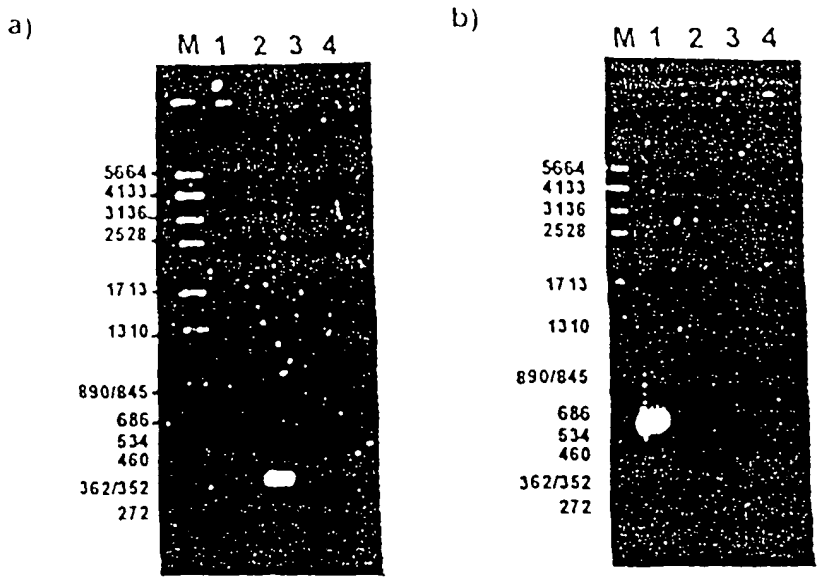


Fig. 3-31: RT-PRC after Passage of Recombinant Viruses
After the transfection and infection of B82 cells with FPV virus and passage twice on MDCK cells, an RT reaction was performed after isolation of all cell RNA. After a PCR reaction, an aliquot of the reaction preparation was electrophoretically separated on an agarose gel.

a) Detection of RNA molecules that have the genetic information for CAT (pHL1510)
Lane 1: For the PCR reaction, an aliquot of an RT reaction preparation without the addition of RT was used; the oligonucleotides #T3-NTR and #CAT-RTPCR were used. Lane 2: RT-PCR reaction results with the oligonucleotides #T3-NTR and #CAT-RTPCR. In Lane 3, only #T3-NTR was used; in Lane 4, only #CAT-RTPCR was used in the RT-PRC reaction.

b) Detection of recombinant RNA that encodes for the nucleoprotein (pHL1563): Lane 1: RT-PCR reaction results with the oligonucleotides #T3-NTR and #NP554. Lane 2: For the PCR reaction, an aliquot of the RNA preparation without the addition of RT was used and the oligonucleotides #T3-NTR and #NP554 were used. In Lane 3, only #T3-NTR was used; in Lane 4, only #NP554 was used in the RT-PRC reaction.

3.13 Generation of Cloning Vectors with the Murine RNA-Polymerase I Transcription System

Through the insertion of foreign sequences within the end regions of the influenza segment 5, the CAT expression level was reduced only slightly. The detection of the expression of a recombinant influenza segment with terminal foreign sequences is also possible. This demonstrates that the RNA polymerase I transcription system with the modified segment ends is suitable both for heterologous and homologous influenza-mediated gene expression. Consequently, it is of interest for additional applications to improve the plasmid systems such that the inserted subterminal sequence sections contain a series of restriction cuts that enable

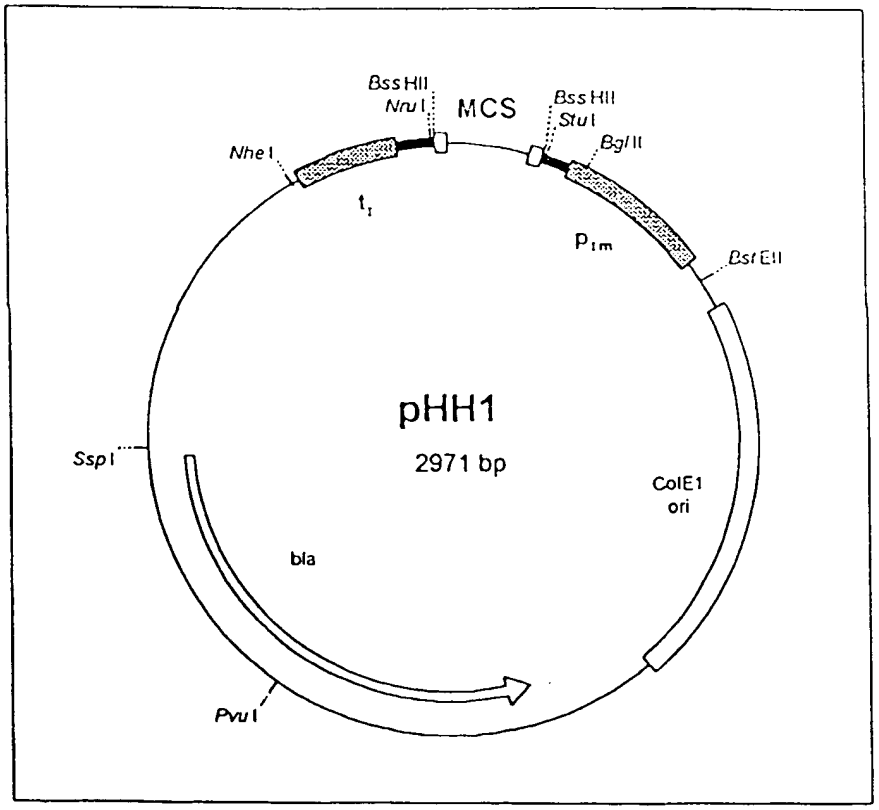


Fig. 3-32: Structure of the Cloning Vector pHH1
The plasmid includes the murine RNA polymerase I promoter (p_{Im}) and terminator (t_I). The cDNA for the noncoding regions of segment 5 are depicted as heavy black lines. A multiple cloning site (MCS) that enables the insertion of any fragments is introduced between the flanking influenza-specific sequences.

the introduction of DNA fragments by simple cloning steps.

The plasmids pHL1509, pHL1510, pHL1511, and pHL1512 were cleaved with *Hin*dIII. After religation of the vector fragment including 3001 bp, the plasmids pHL1559, pHL1560, pHL1561, and pHL1562, respectively, were obtained. To obtain plasmids that contain no recognition sequences for the restriction enzymes *Bam*HI, *Xba*I, *Pst*I, and *Not*I in the vector portion, but instead have only a single recognition sequence in the multilinker region, the *Sca*I fragments including 1985 bp in each case were isolated and ligated with a 986-bp-long *Sca*I fragment from pHL1643 (In pHL1643, the relevant cuts in the vector were removed by cleaving the plasmid pHL1324 with *Not*I/*Bam*HI and subsequent filling with Klenow polymerase). The resultant plasmids were named pHH1, pHH2, pHH3, and pHH4.

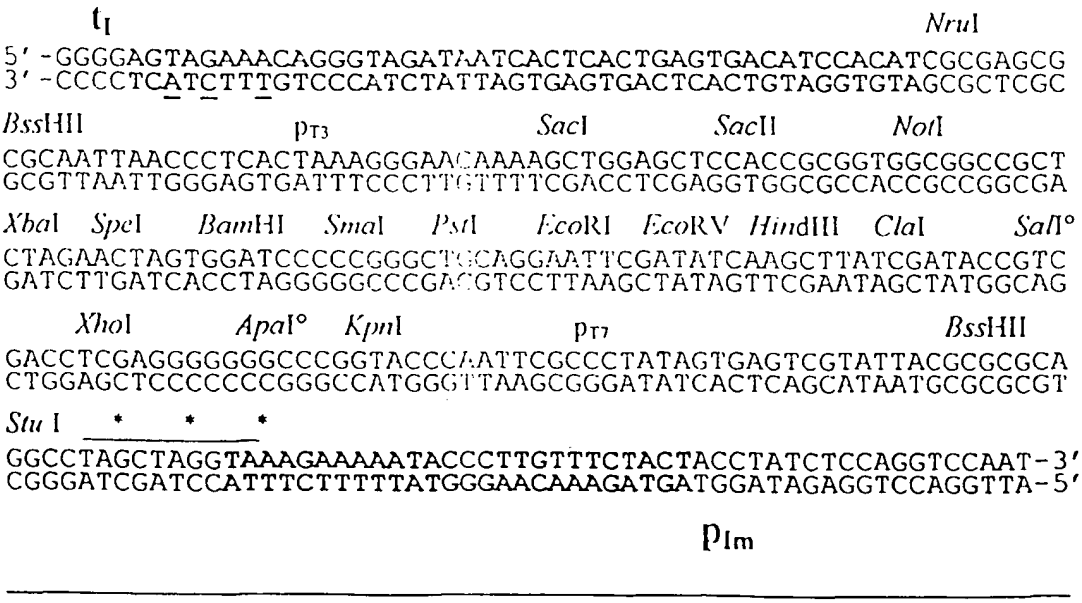


Fig. 3-33: The Multiple Cloning Region of the Cloning Vector pHH1
The influenza-specific vRNA end sequences of segment 5 (bold) are located between a murine rDNA promoter and rDNA terminator. A multiple cloning site (from pBlueScriptII KS) that enables the insertion of any fragments by simple cloning steps is inserted inside these flanking regions. Recognition sequences identified with ° are present more than once in the plasmid vector. The asterisks (*) indicate the position of three stop codons in three different reading frames.

As an example of the plasmids constructed, the construct pHH1 is presented in Fig. 3-32 and its multiple cloning sequence is presented in Fig. 3-33. The cDNA for the noncoding region of segment 5 is located between a murine rDNA promoter and rDNA terminator. A multiple cloning sequence originating in the cloning vector pBlueScriptII KS (Stratagene) is inserted between the influenza-specific sequences.

In addition to numerous recognition sequences for restriction endonucleases, the promoters for the RNA polymerases from the bacteriophages T3 and T7 are localized in the multilinker section. Thus, it is also possible to produce specific RNA probes after the insertion of a fragment by means of *in vitro* transcription. These probes may be used to detect the synthesis of recombinant RNA molecules after transient transfection and passage. In the vector construct pHH1, the multiple cloning site is present in "SK orientation[1]"; the plasmid pHH2 includes this sequence in opposing "KS orientation". The constructs based on pHH1 or pHH2 result, after transfection, in primary transcripts with vRNA polarity.

In contrast, in the plasmids pHH3 and pHH4, the 5' and 3' cDNA sequences are anchored in antigenomic orientation. These vectors are suitable to generate constructs that result, after transfection, in the synthesis of cRNA. pHH3 contains the multiple cloning sequence in "SK orientation"; in pHH4, the opposing "KS orientation" is present.

The plasmid system produced thus enables the cloning of foreign genes both in vRNA and in cRNA orientation. Through the exactly identical structure, the efficiency of the RNA synthesis can be determined for both orientations.

[1] derived from the position of the flanking restriction endonucleases *Sac*I and *Kpn*I

3.14 Establishing the Human RNA-Polymerase I Transcription System

The RNA polymerase I transcription system with the murine rDNA promoter results in a high transcription rate only in mouse cells because of the "species-specificity" of the murine RNA polymerase I initiation complex. An application of the RNA polymerase I system in primate cells as well should be possible through the use of the human rDNA promoter.

3.14.1 Generation of the Expression Constructs

As a plasmid with a human promoter, the plasmid $pHrP_2$ (generously made available by Dr. I. Grummt, Heidelberg) was used. A 229-bp *Bam*HI/*Hin*cII and a *Hin*cII/*Hin*fI fragment including 152 bp was isolated from this plasmid. These fragments were inserted together with the oligopair #PolI-Oligo1[24]/#PolI-Oligo2[25] in the vector pOM8 cleaved with *Bam*HI/*Pst*I. The resultant construct was named pHL1810. To verify the proper structure, the region of the promoter was sequenced. The sequencing yielded deviations from published sequences (Financsek *et al.*, 1982; Miesfeld & Arnheim *et al.*, 1982). The human promoter sequence from the position -1 through -407 (*Sau*3A-cut) is presented in the following:

```
                  5'-GATCCTT TCTGGCGAGT CCCCGTGCGG AGTCGGAGAG CGCTCCCTGA
                  3'-CTAGGAA AGACCGCTCA GGGGCACGCC TCAGCCTCTC GCGAGGGACT

-360 GCGCGCGTGC GGCCCGAGAG GTCGCGCCTG GCCGGCCTTC GGTCCCTCGT GTGTCCCGGT
     CGCGCGCACG CCGGGCTCTC CAGCGCGGAC CGGCCGGAAG CCAGGGAGCA CACAGGGCCA

-300 CGTAGGAGGG GCCGGCCGAA AATGCTTCCG GCTCCCGCTC TGGAGACACG GGCCGGCCCC
     GCATCCTCCC CGGCCGGCTT TTACGAAGGC CGAGGGCGAG ACCTCTGTGC CCGGCCGGGG

-240 CTGCGTGTGG CACGGGCGGC CGGGAGGGCG TCCCCGGCCC GGCGCTGCTC CCGCGTGTGT
     GACGCACACC GTGCCCGCCG GCCCTCCCGC AGGGGCCGGG CCGCGACGAG GGCGCACACA

-180 CCTGGGGTTG ACCAGAGGGC CCCGGGCGCT CCGTGTGTGG CTGCGATGGT GGCGTTTTTG
     GGACCCCAAC TGGTCTCCCG GGGCCCGCGA GGCACACACC GACGCTACCA CCGCAAAAAC

-120 GGGACAGGTG TCCGTGTCGC GCGTCGCCTG GGCCGGCGGC GTGGTCGGTG ACGCGACCTC
     CCCTGTCCAC AGGCACAGCG CGCAGCGGAC CCGGCCGCCG CACCAGCCAC TGCGCTGGAG

-60  CCGGCCCCGG GGGAGGTATA TCTTTCGCTC CGAGTCGGCA TTTTGGGCCG CCGGGTTATT-3'
     GGCCGGGGCC CCCTCCATAT AGAAAGCGAG GCTCAGCCGT AAAACCCGGC GGCCCAATAA-5'
```

[24] #PolI-Oligo1: 5'-AGTCGGCATTTTGGGCCGCCGGGTTATTGGTGAGACGCTGCA-3'

For the nucleotidelike binding of the influenza end sequences to the human promoter sequence and to the murine terminator sequence, it was necessary to generate intermediate constructs: A *Bam*HI/*Pst*I fragment from pHL1810 including 421 bp was inserted into the vector pHH2 cleaved with *Bam*HI/*Pst*I. This construct (pHL1811) was cleaved for the removal of the murine promoter with *Bst*EII and *Bam*HI and the ends filled in with Klenow polymerase. The resultant 2986-bp-long plasmid is called pHL1812. After cleavage of this construct with *Kpn*I/*Eco*RV, a PCR fragment cleaved with *Kpn*I was inserted.

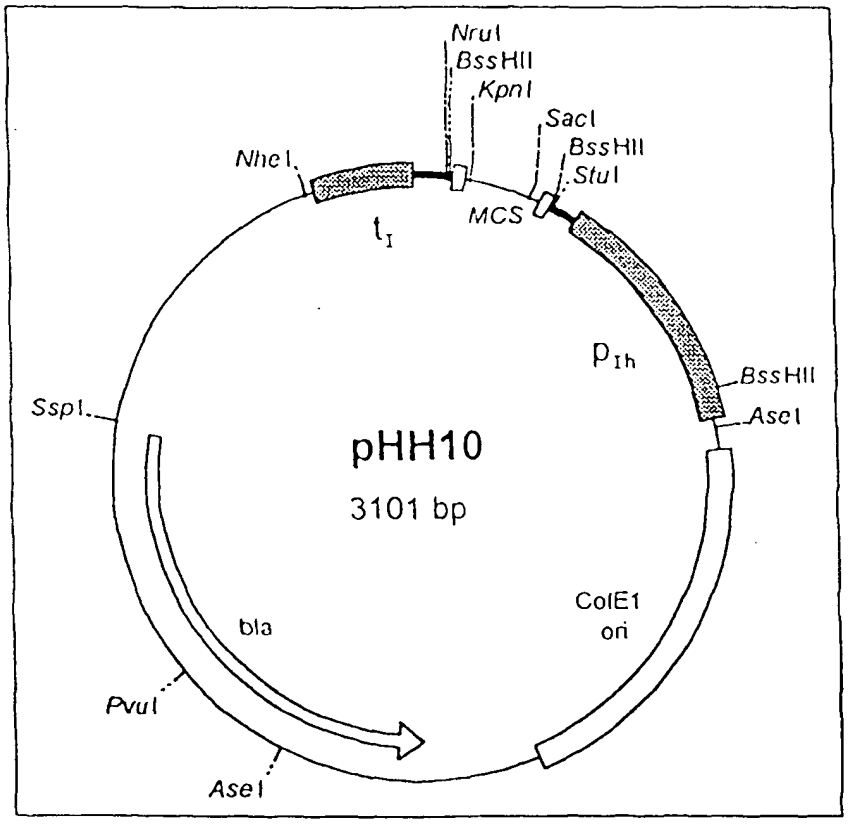


Fig. 3-34: The Plasmid pHH10 with Human rDNA Promoter
The plasmid contains a polymerase I transcription unit with the human rDNA promoter (p_{Ih}) and murine terminator (t_I). The cDNA for the noncoding region of segment 5 is depicted as a heavy black line. A multiple cloning site, which enables the insertion of any fragments, is inserted between the flanking influenza-specific sequences.

[25] #PolI-Oligo2: 5'-GCGTCTCACCAATAACCCGGCGGCCCAAAATGCCG-3'

For the amplification of this PCR fragment, the oligonucleotides #PrEH40[26] and #T7[27] were used. pHH2 was used as the template. After cleavage of the resultant plasmid (pHL1813) with *Bsm*BI and religation, two constructs were characterized by sequencing. pHH10 includes, at position +1, an adenosine residue corresponding to the influenza-vRNA-5'-end (see Fig. 3-34), whereas pHH11 has a guanosine residue at this position, which corresponds to the transcription start of human ribosomal RNA.

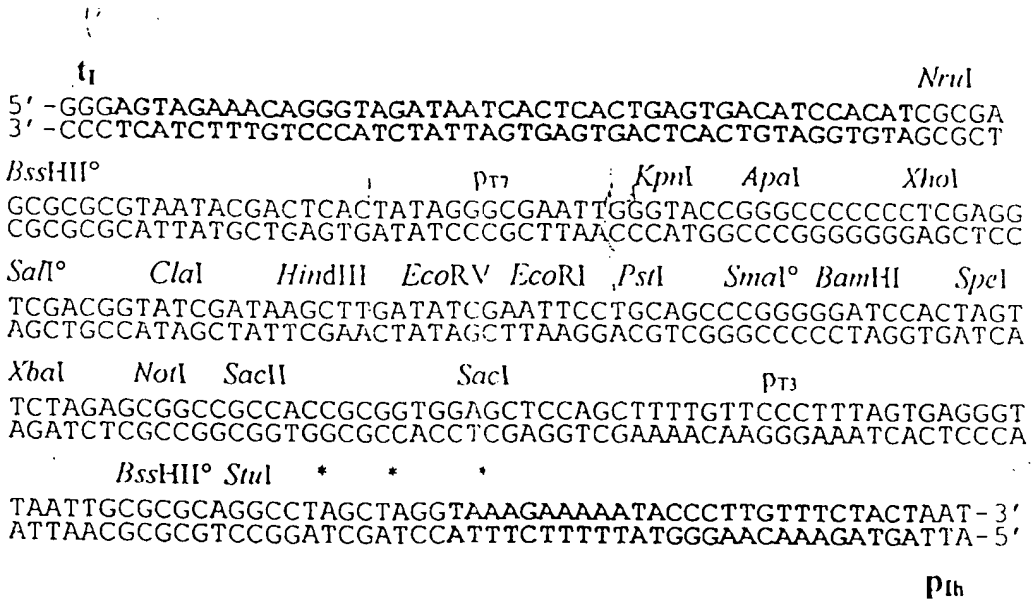


Fig. 3-35: The Multiple Cloning Region of the Vector pHH10
The influenza-specific end sequences of segment 5 (bold) are located between human rDNA promoters and a murine terminator. A multiple cloning sequence (from pBlueScriptII KS) that enables the insertion of any fragments by simple cloning steps is inserted inside these flanking regions. Recognition sequences identified with ° are present more than once in the plasmid vector. The asterisks (*) indicate the position of three stop codons in three different reading frames. The plasmid pHH20 (not shown) contains the multiple cloning sequence in the opposing orientation, analogous to the vector pHH1 (Fig. 3-33) with a murine rDNA promoter.

[26] PrEH40: 5'-ATCGAATTCGTCTCCTTAGTAGAAACAAGGGTATT-3'

[27] #T7: 5'-GTAATACCGACTCACTATAGGGC-3'

3.14.2 Detection of the *in-vivo* RNA Synthesis by CAT-Analysis

The plasmid vectors pHH10 and pHH11 were linearized with *Hin*dIII. The insertion of a 787-bp *Hin*dIII fragment with the sequence for the reporter gene CAT from pcDNA3CAT yielded the plasmids pHL1844 and pHL1845 (see Fig. 3-36). These constructs include the human RNA polymerase I promoter as well as the murine terminator sequence. The end sequences of the vRNA segment 5 with the optimized promoter sequence are located between these elements. The inserted reporter gene CAT enables the detection of the RNA transcription *in vivo* after infection with helper viruses. The construct pHL1844 contains, at the transcription start, an adenosine residue, as corresponds to the 5' end of the vRNA. At this position, pHL1845 has a guanosine residue, which corresponds to the wildtype situation of the transcription start of human ribosomal pre-RNA (Fig. 3-36).

After transfection of the constructs pHL1844 and pHL1845 on COS-7 cells, infection with influenza helper viruses took place 24 hours later. After an additional 16 hours, the secondary infection of MDCK cells with the supernatant of the primarily infected COS-7 cells occurred.

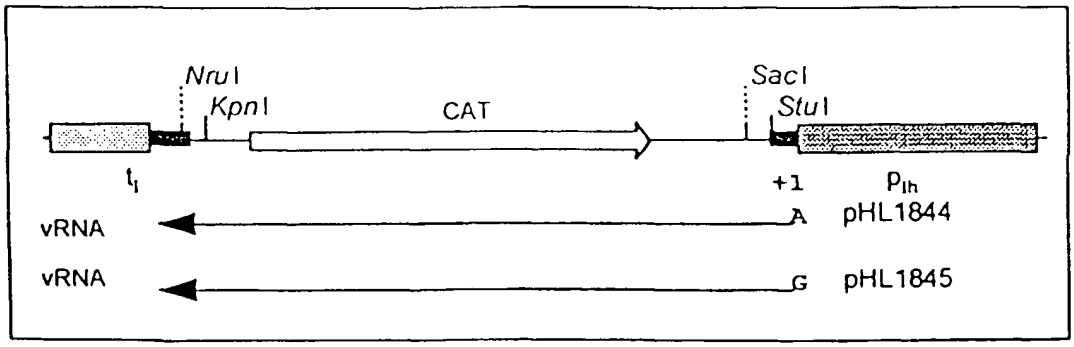


Fig. 3-36: Structure of the Reporter Constructs with a Human rDNA Promoter

The upper part depicts the basic structure of the constructs for transfection of primate cells. The RNA polymerase transcription unit includes the human rDNA promoter (p_{Ih},-1 through -407) and the murine terminator (t_I). The influenza sequences of segment 5 with the optimized promoter sequence on the 3' end of the vRNA are located between these rDNA transcription elements. The vRNA molecules formed after transcription are symbolized by the arrows. In the construct pHL1844, an adenosine residue is located at position +1 of the transcript produced, which corresponds to the wildtype sequence of segment 5. pHL1845 has at this position a guanosine residue corresponding to the transcription start of human pre-rRNA.

Every 16 hours after infection, the cells were processed and the CAT reaction performed (see Fig. 3-37). Both variants showed a high CAT activity both in the primary infection and in the secondary infection.

The equally high activity of the two constructs investigated, which differ from each other only at the position +1, shows that the efficiency of the initiation of the RNA polymerase I transcription at position +1 is not altered by the substitution of a guanosine residue by an adenosine residue. The high CAT expression of pHL1845 from the primary transfection as from the passage documents, on the other hand, that the replication and packaging of the viral RNA is not reduced by this nucleotide exchange at position 1.

For a comparative investigation as to whether the increased reporter gene activity is also

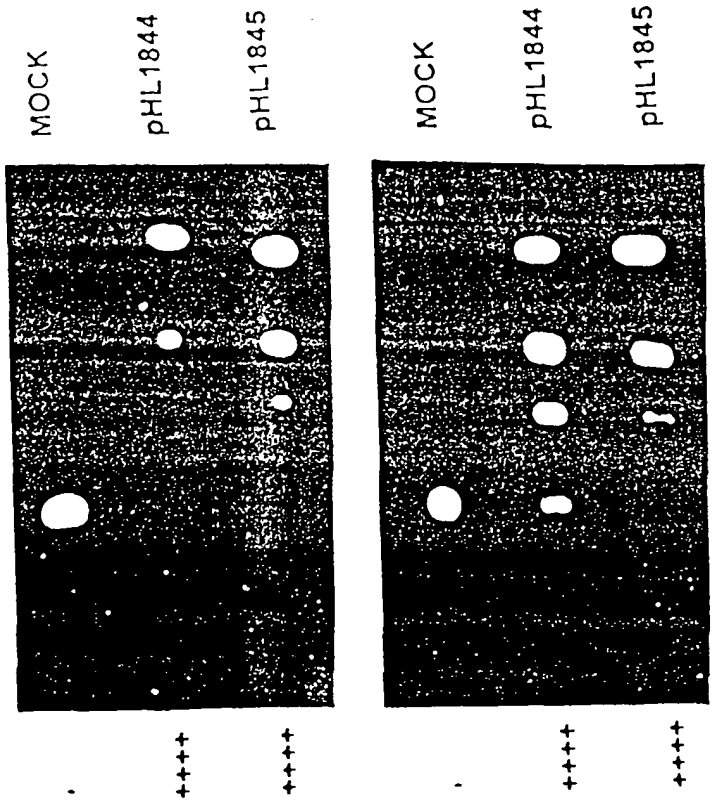


Fig. 3-37: CAT Analysis for the Detection of *in vivo* Synthesized Transcripts by the Human RNA Polymerase I Transcription System
On the left, CAT analyses after transfection and the infection of COS-7 cells are shown; on the right, the CAT reaction results after passage on MDCK cells are shown. 10^7 COS-7 cells each were transfected with the plasmids pHL1844 and pHL1845, resp ctively (5 µg DNA/30 µl lipofectamine). 24 hours after transfection, the viral infection was carried out for 60 minutes at 37°C (A/Asia/5/57). After 16 hours, an aliquot of the cell supernatant was placed on MDCK cells. The cells were processed in each case 16 hours after infection and the CAT reaction performed.

detectable in the human transcription system as a result of the three nucleotide exchanges on the 3' end of the vRNA, the construct pHL1863 with wildtype vRNA ends was generated (ligation of a 2093-bp *Pvu*I/*Nco*I fragment from pHL1844 with a *Pvu*I/*Nco*I fragment from pHL1168 including 1703 bp).

After transfection of pHL1863 and pHL1844 on COS-7 cells with subsequent influenza infection, the CAT reaction was performed 16 hours after the infection. The results are presented in Fig. 3-38. The construct with wildtype vRNA ends has, as with the murine rDNA promoter, only a weak CAT signal, roughly 1/20 of the reaction product compared to the construct with the optimized promoter sequence (pHL1844).

In another preparation, it was investigated whether, besides the monkey cells COS-7, other

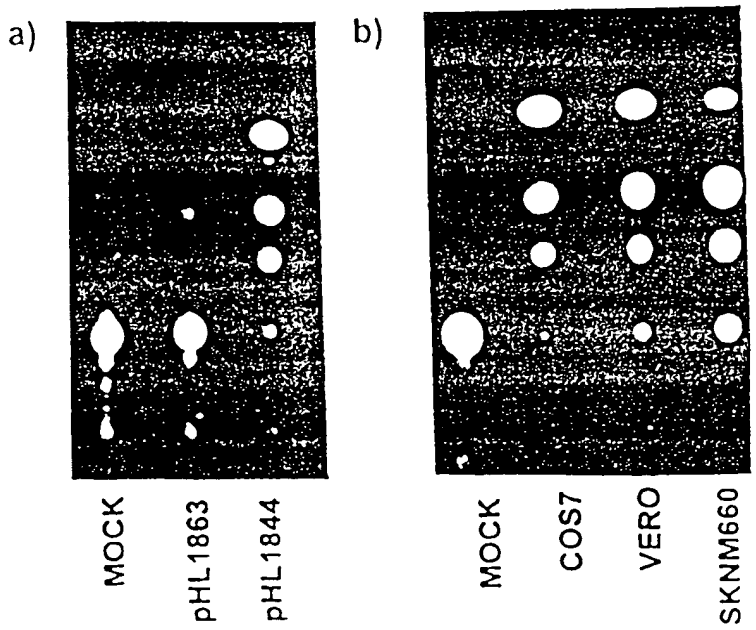


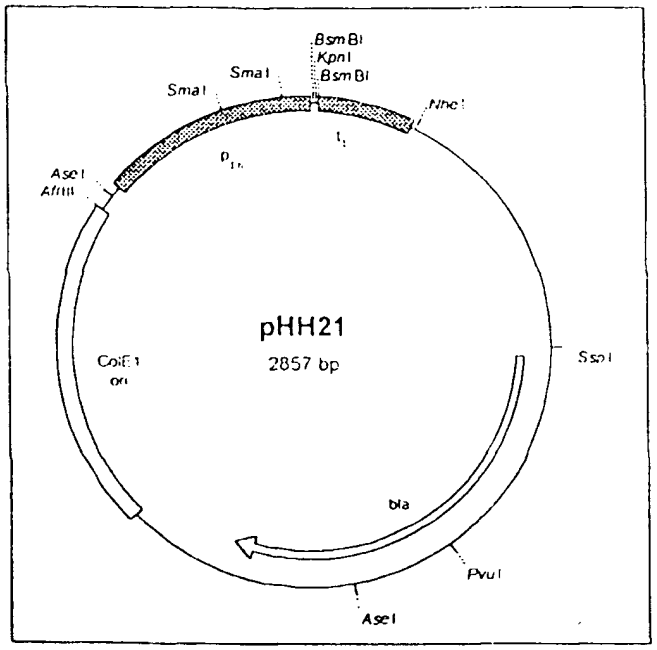
Fig. 3-38: vRNA-Sequence-Specificity and Cell-Specificity of the Human rDNA Promoter
a) Comparison of the CAT reaction results between pHL1836 with wildtype ends and pHL1844 with three nucleotide exchanges on the 3' end of the vRNA after transfection of COS-7 cells.

b) CAT reaction after transfection of the construct pHL1844 in the cell lines COS-7, Vero, and SKNM cells.

In each case, the viral infection (A/FPV/Bratislava) took place 24 hours after transfection (5 μg plasmid DNA/30 μl lipofectamine). The cells were processed 16 hours after the infection and the CAT reaction performed with the cell lysate.

cell types also have CAT activity after transfection with pHL1844 and subsequent infection. In the CAT reaction, high CAT activity is detectable in the monkey kidney cell line Vero as well as in the human cells SKNM660 (see Fig. 3-38) and HeLa cells (not shown).

The cloning vector pHH21 (see Fig. 3-39) was constructed for the site insertion of cDNA sequences between the human rDNA promoter and the murine terminator. This vector permits the construction of reporter plasmids both for the selective mutagenesis with influenza A viruses and for other negative strand RNA viruses (such as Borna viruses).



*Bsm*BI *Kpn*I *Bsm*BI

5′-GGCCGCCGGGTTATTGGAGACGGTACCGTCTCCTCCCCCCCAACTTCGGA-3′
3′-CCGGCGGCCCAATAACCTCTGCCATGGCAGAGGAGGGGGGGTTGAAGCCT-5′

p_{Ih} t_I

Fig. 3-39: The Cloning Vector pHH21
The plasmid pHH21 includes two recognition sequences for the "bracket enzyme" *Bsm*BI. The sequences that form the vector component after cleavage with *Bsm*BI are bolded. Since the overhanging ends cannot be hybridized, this results in a high yield of recombinant plasmid molecules after insertion of DNA fragments between the human rDNA promoter (p_{Ih}) and the murine terminator (t_I).

3.15 Detection of the Influenza A-Mediated Expression of the Green Fluorescing Protein

The green fluorescing protein (GFP) from the jellyfish *Aequorea victoria* is well-suited for the analysis of biological processes in the living cell. The expression in the cells can be detected either by fluorescence microscopy or by FACS flow cytometry.

The encoding sequence for the GFP protein was isolated by cleavage from TU#65 (Chalfie *et al.*, 1994) with *Kpn*I and *Eco*RI and inserted in the murine RNA polymerase I vector pHL1512 (*Kpn*I/*Eco*RI). The resulting product was called pHL1565. After transfection of B82 cells and subsequent infection with FPV virus, no expression could be detected by fluorescence microscopy. This negative result was probably caused by the relatively weak fluorescence of the wildtype GFP.

Consequently, new GFP reporter constructs that contain mutations in the chromophoric group and result in greatly increased fluorescence were generated (Heim *et al.*, 1995). An increase in the expression rate should also be obtained through the use of the human RNA

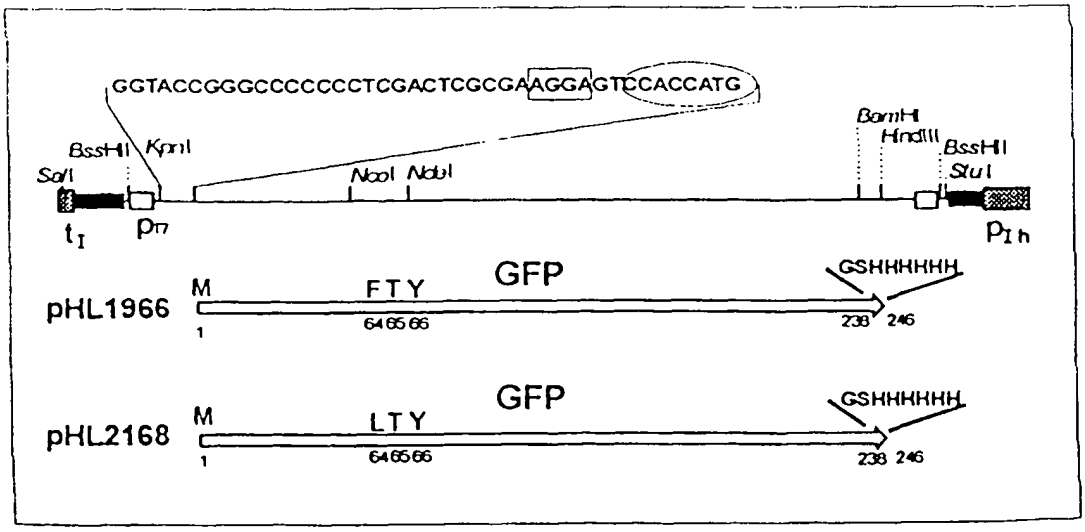


Fig. 3-40: Generation of the Plasmids for GFP Expression
Compared to the wildtype GFP, with pHL1966, the serine at position 65 is replaced by a threonine residue; in pHL2168, the amino acid 64 phenylalanine is also replaced by a leucin. On the C-terminal end, inside eight additional amino acids, there are six histidine residues. To improve the translation start, a Kozak sequence (CCACCATGA; Kozak, 1980) was inserted.

polymerase I promoter. The generation of the expression constructs is presented in Fig. 3-40. Based on the vector pHH10, the plasmids pHL1966 and pHL2168 contain the GFP reading frame. In comparison with the wildtype GFP, with pHL1966, the serine at position 65 is replaced by a threonine residue; in pHL2168, the amino acid 64 phenylalanine is also replaced by a leucin. On the C-terminal end, besides two additional amino acids (glycine, serine), six histidine residues are attached.

For the transfection of approximately 10^7 COS-7 cells, 5 μg plasmid DNA and 20 μl lipofectamine each were used. The negative control was incubated with lipofectamine without DNA and incubated, instead, with medium only. After a three-hour incubation, the lipofectamine/DNA mixture was washed from the cells. The further incubation now took place for 24 hours with DMEM medium. The viral infection with FPV virus was carried out for 60 minutes at 37°C. Sixteen hours after infection, the cells were washed again with PBS and overlayered with 1 ml PBS. The expression of the GFP protein was then investigated by means of fluorescence microscopy. The cells of the negative control presented only a slight green background fluorescence. In contrast, in the preparations with the transfected plasmid molecules, green tinted cells were clearly discernible (cf. Fig. 3-41). An aliquot of

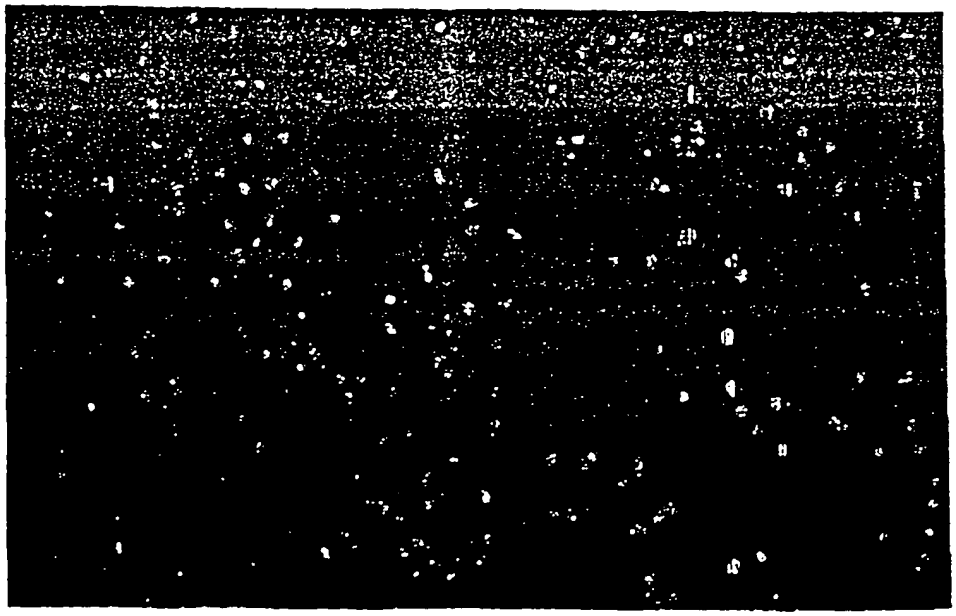


Fig. 3-41: Detection f th GFP Expr ssi n by Flu resc nce Micr sc py
COS-7 cells were transfected with the construct pHL1966. The infection with FPV virus occurred after 24 hours. The cells were photographed 16 hours after infection.

the cell supernatant was passaged on MDCK. After 16 hours, green cells were also detectable here.

The hybrid vRNA encoding for the GFP was thus synthesized after transfection *in vivo* and was translated after infection by viral GFP-mRNA into the GFP protein. The protein quantity formed as well as the proportion with correct folding of the protein was adequate to be able to detect the reporter gene in fluorescence microscopy.

For more precise determination as to how high the proportion of GFP-expressing cells is in the total cell population, FACS analyses were performed. In this process of fluorescence activated cell sorting, the fluorescence emission of each individual cell is determined. After excitation of an individual cell with the monochromatic light of an argon laser (488 nm), the green light emitted is detected. Using the software "Cellquest", a histogram plot is recorded. The cell count is entered on the y-axis; the calculated dimension of the individual intensity of the emitted light is reported on the x-axis.

The results presented in Fig. 3-43 came from the following experiment: For the

Fig. 3-42: D t ction f th GFP Expression Eight Hours after the Primary Influenza Infection
COS-7 cells were transfected with the construct pHL1966. The infection of the transfected cells with FPV virus occurred after 24 hours. Eight hours after infection, the cells were photographed.

transfection of 10^7 COS-7 cells, 5 μg pHL1966 or pHL2168 plasmid DNA was used in each case. The lipofectamine/DNA mixture was left on the cells for 3 hours and then replaced by DMEM medium. The infection with FPV virus occurred after 24 hours. After an additional 16 hours of incubation, an aliquot of the cell supernatant was passaged on MDCK cells.

The infected cells were washed with PBS in each case 16 hours after the primary infection or secondary infection, detached with PBS (+trypsin), and diluted to a cell concentration of approximately 10^5 cells/ml in PBS. Until the FACS analysis, the cells remained in polystyrene tubes on ice. In the flow cytometry, 10^4 cells were analyzed per batch in each case. In order to determine the proportion of autofluorescence of the cells, transfected and noninfected COS-7 and MDCK cells were used (cf. Fig. 3-43 A). The fluorescence emissions can be broken down into two ranges: The level of cellular autofluorescence can reach the maximum value of 2×10^2 units. Consequently, fluorescence intensities above this range result from the GFP fluorescence. Through the definition of an increased base value of 6×10^2 units, it is guaranteed that only cells clearly expressing GFP are considered in the evaluation.

In the primary infection, the proportion of fluorescent cells after transfection with pHL1966 was 6.3%; with pHL2168, 4.5% of the cells have fluorescence (see Fig. 3-43 B). In the secondary infection, the proportion of GFP-expressing cells with pHL1966 was 41.3%; with pHL2168, this proportion stood at 54.3%.

In further experiments, the fluorescence of the GFP protein was determined at different points in time. It was demonstrated that with both fluorescence microscopy and FACS analysis, GFP-expressing cells were already detectable 4 hours after infection.

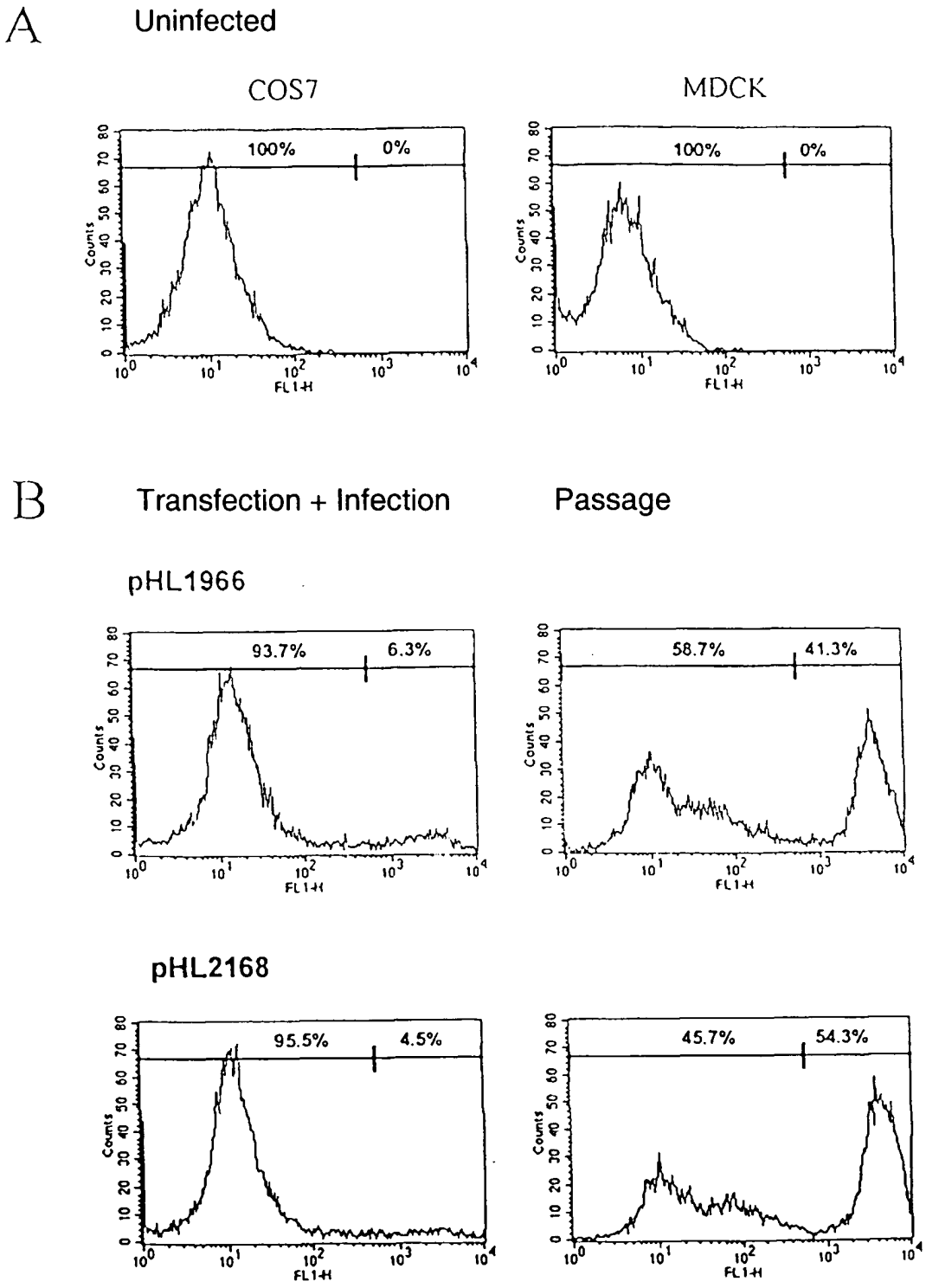


Fig. 3-43: D termination f GFP-Expr ssing Cells by FACS Fl w Cytometry
A Determination of the cellular autofluorescence of COS-7 and MDCK cells
B On the left, the results after transfection with pHL1966 and pHL2168 and infection with FPV virus on COS-7 cells are shown; on the right, the results, after passage on MDCK cells.

3.16 Generation of a "PolI and/PolII Double Transcription System" for the Synthesis of vRNA and mRNA

The lifecycle of negative-strand RNA viruses includes the process of replication, which results in the amplification of viral RNA molecules, as well as the process of transcription, in which the viral mRNA is formed. The translation of the mRNA in the cytoplasm then results in the expression of viral proteins. Using the RNA polymerase I (PolI) transcription system, the viral RNA can be formed nucleotidelike by the cellular RNA polymerase I and the associated transcription factors/termination factors. MRNA expression alone and with it the synthesis of the corresponding proteins, but not the associated vRNA molecules, can occur using RNA polymerase II (PolII) expression systems. In this, a cDNA sequence is inserted between an RNA polymerase II promoter and a polyadenylation signal sequence (pA-signal).

Consequently, it was of interest to investigate whether it is possible to integrate an open reading frame into the two available RNA polymerase systems such that both the formation of viral RNA and synthesis of mRNA and proteins occur. The formation of viral RNA can be detected as follows: After transfection and subsequent infection, only viral or pseudoviral RNA molecules, i.e., with influenza-specific ends, can be packaged. Through successful passage of the viruses and subsequent CAT resynthesis, the formation and packaging of viral RNA can be detected. The synthesis of mRNA and protein can be determined directly after transfection and without infection with helper viruses.

3.16.1 Generation of the Expression Constructs

The plasmid pHL1325 was cleaved with *Hin*dIII/*Xho*I and ligated with the two oligonucleotides #LiHX1[28] and #LiHX1[29]. The resultant plasmid pCMV1 includes a

[28] #LiHX1: 5'-AGCTGTTAACGCTAGCGCGCGTCTCCCATGGTGACCT-3'

[29] #LiHX2: 5'-TCGAGGTCACCATGGGAGACGCGCGCTAGCGTTAAC-3'

polymerase II promoter and a pA signal, as well as the multiple cloning region between these sequence elements. The plasmid pHL1993 resulted from the ligation of a 3.3-kb *Afl*III/*Bam*HI and a 329-bp *Bgl*I/*Bam*HI fragment from pHL1844 with the oligonucleotide pair #LiAB1[30]/#LiAB2[31]. Compared to pHL1844 , this construct contains a truncated human promoter sequence (position -223 through -1, referenced from the transcription start point).

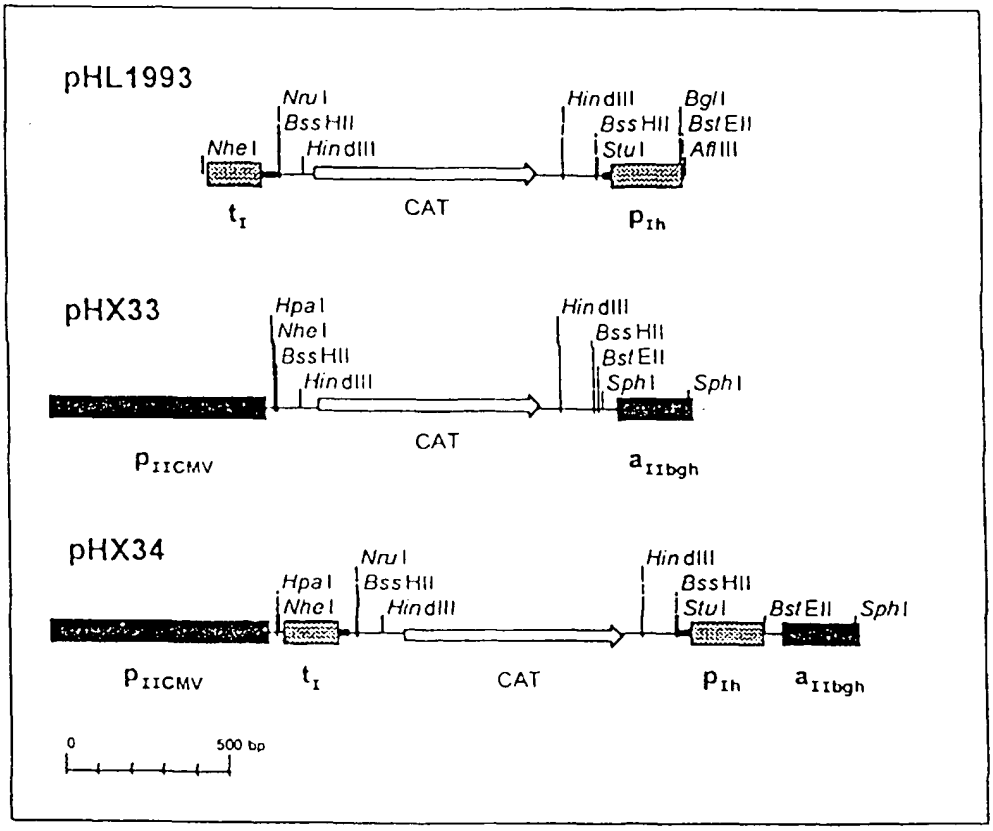


Fig. 3-44: Structure of the PolI/Pol II Double Transcription System
The plasmid pHX34 includes an RNA polymerase I transcription unit, which contains the reporter gene CAT with influenza end sequences (segment 5) from pHL1993. The entire RNA polymerase I transcription unit is surrounded by an RNA polymerase II transcription system with promoter (immediate early promoter of the human cytomegalovirus, p_{IICMV}) and polyadenylation sequence (from the gene for the bovine growth hormone, a_{IIBGH}). There are two different RNA polymerase promoters in opposing orientation on the plasmid, which result, however, in the transcription of two mRNAs with the same reading frame, i.e., either directly via mRNA molecules in *sense*-orientation (RNA polymerase II promoter) or indirectly via vRNA in *antisense*-orientation (RNA polymerase I promoter).

[30] #LiAB1: 5'-CATGTGGTGACCGCCGGGA-3'

[31] #LiAB2: 5'-CGGCGGTCACCA-3'

The construct pCMV1 was linearized with *Bss*HII and ligated along with a *Bss*HII fragment (960 bp) from pHL1993. The resultant product with the desired orientation was named pHX33 (Fig. 3-44). The insertion of an *Nhe*I/*Bst*EII fragment including 1464 bp into the vector pCMV1 cleaved with the same enzymes resulted in the construct pHX34. Compared to pHX33, this plasmid contains a complete RNA polymerase I transcription unit framed by an RNA polymerase II promoter ("immediate early" promoter of the human cytomegalovirus) and a polyadenylation signal (from the gene for bovine growth hormone BGH).

For the transfection of 10^7 COS-7 cells, 5 μg plasmid DNA and 20 μl lipofectamine were

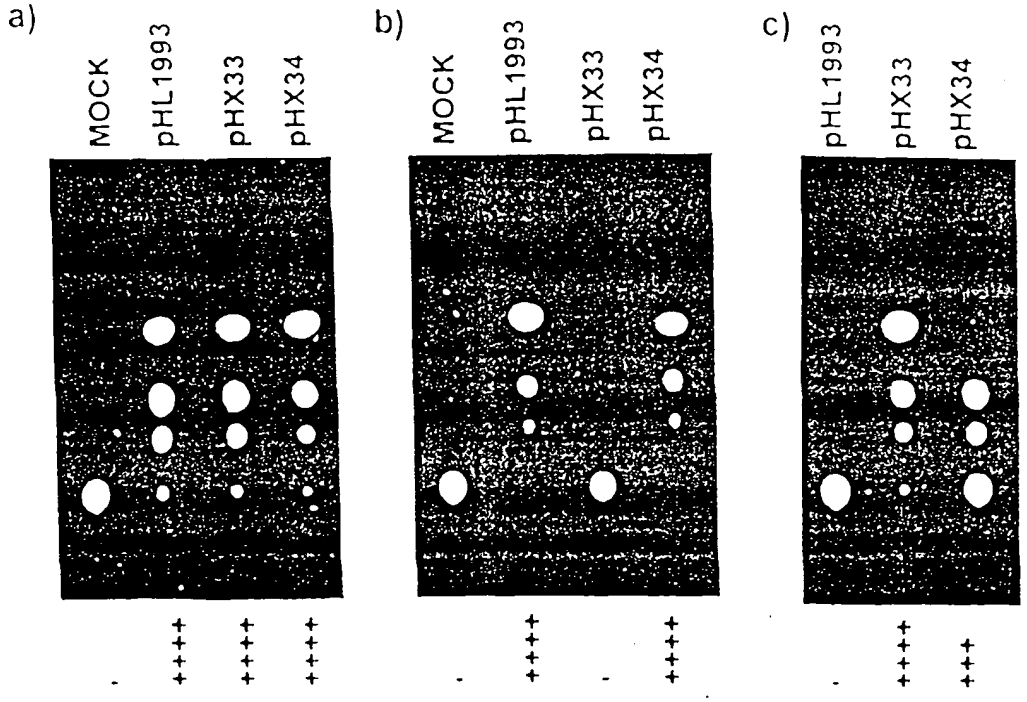


Fig. 3-45: CAT Analysis for the Detection of the *in vivo* Synthesis of vRNA and mRNA in the PolI/Pol II Double Transcription System
a) Transfection of COS-7 cells and subsequent infection with FPV virus. The cells were processed eight hours after infection and the CAT reaction performed.
b) After the passage of the cell supernatant on MDCK cells, the cells were processed 24 hours after infection.
c) Transfection of COS-7 cells (without infection with FPV virus). The cells were processed 48 hours after transfection.

used. The infection with FPV virus was carried out 24 hours after transfection. The passage on MDCK cells took place after 8 hours. The primarily infected cells were processed after 8 hours; the secondarily infected cells, after 24 hours; and the CAT reaction was performed in each case. In the preparations after transfection of COS-7 cells and subsequent infection with FPV virus, high CAT activity was detectable with all constructs (Fig. 3-45a). After passage on MDCK cells, the constructs pHL1993 and pHX34 presented a high CAT signal strength, whereas for the construct pHX34, as anticipated, no CAT activity was detectable (Fig. 3-45b).

In a parallel preparation, COS-7 cells were only transfected but not infected with virus. The cells were processed 48 hours after the transfection. pHX33 and pHX34 presented a strong CAT signal, whereas the construct pHL1993 had no CAT activity. The level of CAT expression was somewhat less with pHX34 than with pHX33 (Fig. 3-45c).

The enzyme activities obtained after transfection and infection result in the construct pHL1993 from the formation of vRNA and the synthesis of mRNA by the viral polymerase complex. The CAT protein quantity that is detected with pHX33 results from the mRNA synthesized by the RNA polymerase II. The positive CAT reaction after passage on MDCK cells with pHX34 verifies the formation of vRNA molecules even with the boxed expression construct. The synthesis of mRNA by the RNA polymerase II is reflected in the CAT activity of this construct in the absence of the influenza infection. The CAT activity obtained with pHX34 in the primary infection should, consequently, result from the translation of two different mRNAs: the direct transcription mediated by the RNA polymerase II and the mRNA formed by the influenza polymerase complex after prior RNA polymerase I synthesis of vRNA.

In summary, it can be noted that the "PolI/Pol II double transcription system" produced with two cellular promoter elements in antisense-orientation results in the *in vivo* synthesis of two

different mRNAs as well as vRNA with the same open reading frame; in addition, the antisense vRNA was formed by the RNA polymerase I.

3.16.2 Deletion of the RNA-Polymerase I Terminators and Promoters

Compared to pHX33, the construct pHX34 has a somewhat lower CAT activity. In order to differentiate as to whether it is the murine rDNA terminator sequence for the human rDNA promoter sequence that reduces the RNA polymerase II-mediated level of expression, these elements were individually deleted from pHX34 (cf. The Fig. 3-44 and Fig. 3-46). The cleavage of the plasmid pHX34 with *HpaI*/*Xho*I and the subsequent filling in with Klenow polymerase yielded the plasmid pHX38, which no longer contains any murine terminator

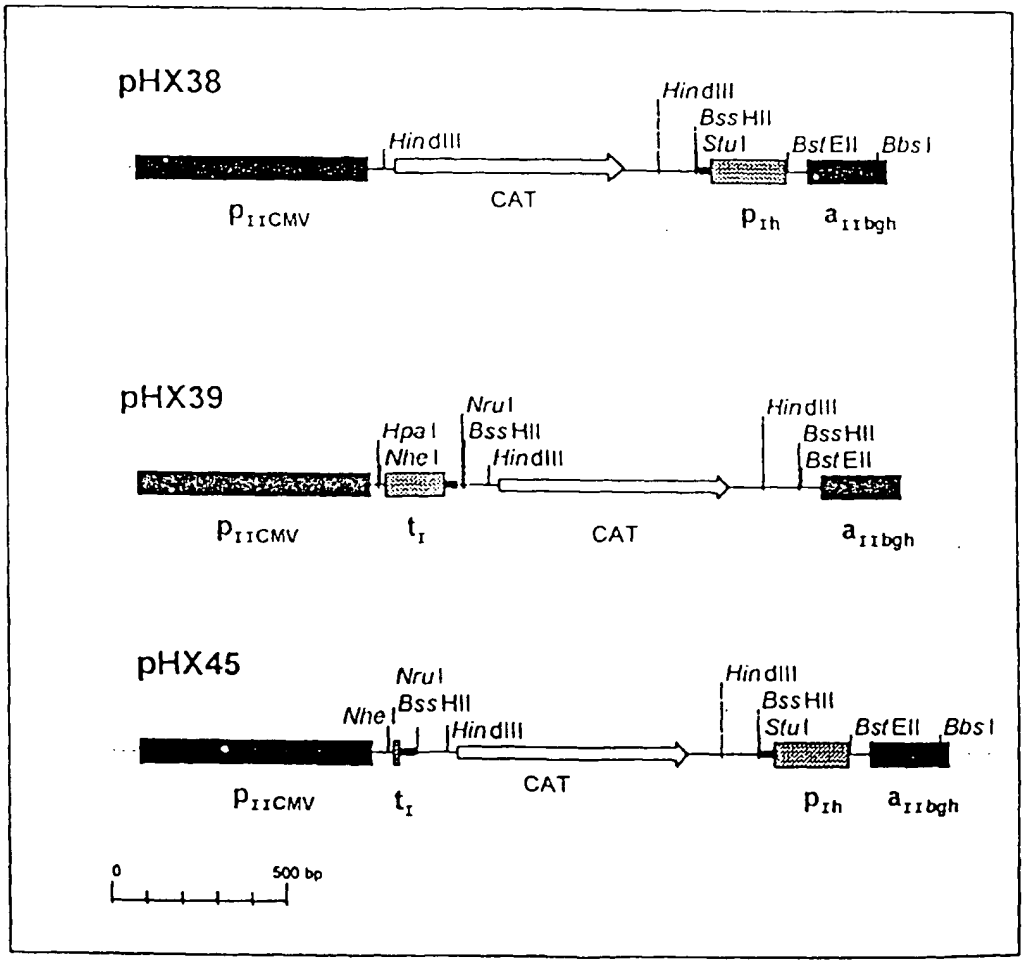


Fig. 3-46: Structure of th C nstructs f r the Diff rentiation f th Sequ nces with Inhibiting Effect on the RNA Polymerase II-Mediated G ne Expr ssion
In the construct pHX38, the murine terminator sequence has been deleted. pHX39 contains the murine terminator but has no human rDNA promoter sequence. With pHX45, the terminator sequence now contains only a single "Sal box"

sequence. The deletion of the human rDNA promoter sequence was achieved by cleavage of pHX34 with *StuI*/*Bst*EII with subsequent Klenow reaction. This plasmid was named pHX39 and contains only the inserted terminator element.

48 hours after transfection (5 μg DNA/20 μl lipofectamine) of 10^7 COS-7 cells, the cells were processed. In the CAT reaction, the signal strength of pHX33 and of pHX38 here is at the same level; in contrast, there is weakened CAT activity with both pHX34 and with pHX39 (cf. Fig. 3-47). Since with these two constructs with a reduced level of expression, the murine terminator region is inserted, it can be concluded from this experiment that the weakened CAT expression results from the insertion of the terminator sequence.

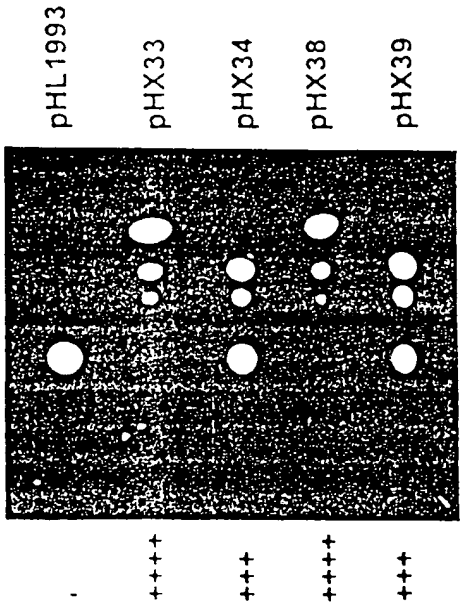


Fig. 3-47: CAT Reaction To Investigate Whether the Murine rDNA Terminator or the Human rDNA Promoter Reduces the RNA Polymerase II-Mediated Expression

After transfection of 10^7 COS-7 cells, the cells were processed after 48 hours and the CAT reaction performed.

3.16.3 RNA-Polymerase I Transcription System With Truncated Terminator

The RNA polymerase II-mediated expression is reduced by the insertion of RNA polymerase I terminator sequences. The reduced level of protein expression could result from the fact that DNA elements result in the binding of cellular factors such as TTF-I, which hinder or terminate overlapping transcription by RNA polymerase II. Another possibility consists in that the inserted terminator sequences reduce the stability of the mRNA, for example, on the RNA level.

Consequently, it should be investigated whether the deletion of subregions of the terminator sequences results in an increase in the RNA polymerase II-mediated gene expression. For this, it first had to be investigated whether the efficiency of the RNA polymerase I-mediated gene expression is affected by the truncation of the terminator region. *In vitro* experiments had shown that, in fact, the so-called "Sal box" including 11 bp is adequate for the termination of the RNA polymerase I transcription, but the efficiency is improved by the authentic sequences between two termination sites (Kuhn *et al.*, 1988). It should be clarified in another set of tests whether *in vivo* only a single "Sal box" is adequate for the termination of the transcription and acts with similar efficiency to the comparative constructs with two "Sal boxes" and flanking sequences.

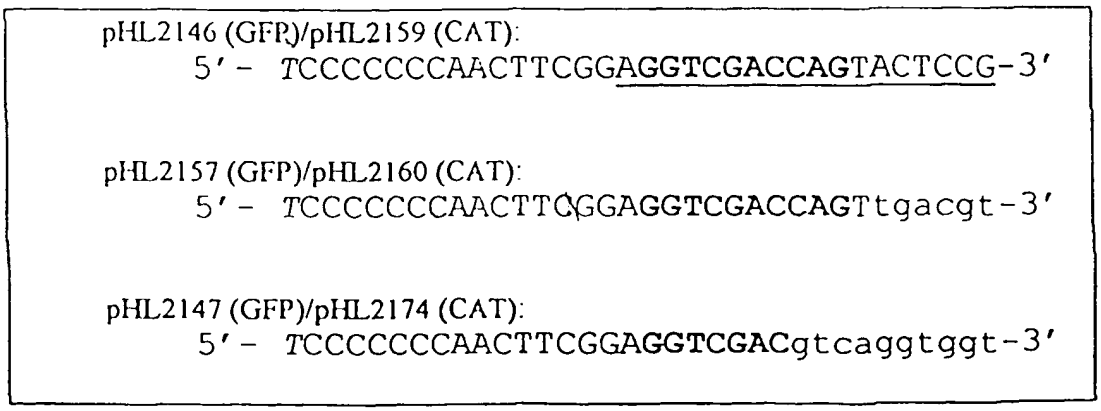


Fig. 3-48: Structure of the Constructs with Truncated Murine Terminator Sections
The italicized T represents the terminal nucleotide (after transcription of a uridine residue on the 3' end of the vRNA) of the influenza sequence. The nucleotides that are part of the "Sal box" (underlined) and that are identical between a human and a murine terminator, are in bold. Vector sequences are represented by lower case letters.

After cleavage of the plasmid pHL1993 with *Nhe*I and *Hin*dIII, a 1048-bp-long *Nhe*I/*Hin*dIII fragment from pHL1966 was inserted in the resultant vector fragment. The resultant construct is called pHL2017 and contains, compared to pHL1966, a truncated human rDNA promoter sequence and the murine rDNA terminator region including 173-bp. A 3459-bp-long *Sal*I/*Nhe*I vector fragment was isolated from the construct pHL2017 and ligated with the oligopair #T1-1[32]/#T1-2[33]. The resultant plasmid was named pHL2146. The construct pHL2146 contains the 18-bp-long Sal-box (see Fig. 3-48). For the generation of the constructs with a "Sal-box" including 12 bp, the oligonucleotide pair #T1-3[34]/#T1-4[35] was used. This plasmid construct, linked with the reporter gene GFP, was called pHL2157. The plasmid pHX35was cleaved with *Sal*I and a 3567-bp-long fragment isolated. The religation of this fragment yielded the plasmid pHL2147. After cleavage, with *Asp*718 and *Pst*I, of the plasmids, which have a GFP reporter gene, an *Asp*718/*Pst*I fragment including 845 bp with an encoding sequence for the reporter gene CAT from pHL1844 was inserted in each case in the resultant vector fragment and the plasmids obtained were called pHL2157, pHL2160, and pHL2174 (cf. Fig. 3-48).

For the transfection of COS-7 cells, 5 μg DNA each (in 15 μl lipofectamine) was used. The cells were processed 16 hours after infection with FPV and the CAT reaction was subsequently performed. The plasmid pHL1844 was used as a reference control. The constructs with the "Sal-box" including 18 bp (pHL2160) and with the "Sal-box" including 12 bp (pHL2157) had a high CAT activity. The signal strength corresponds to the level of the reference construct pHL1844. With the further truncation of the "Sal-box" to eight nucleotides (pHL2174), CAT activity was still detectable. The signal level was, however,

[32] #T1-1: 5'-CTAGCGTCTCACAAAGTTTCGCCCGGAGTACTGG-3'

[33] #T1-2: 5'-TCGACCAGTACTCCGGGCGAAACTTTGTGAGACG-3'

[34] #T1-3: 5'-CTAGCGTCACAACTGG-3'

[35] #T1-4: 5'-TCGACCAGTTGTGACG-3'

significantly lower than with the comparative constructs. After dilution of the cell lysate (1 : 100), no CAT activity could be detected (cf. Fig. 3-49).

In a parallel preparation, with the corresponding terminator variants with GFP as a reporter gene, the fluorescence was determined after 16 hours. In the preparation that was transfected with the variants with eight nucleotides from the "Sal-box" sequence (pHL2147), only a few cells (< 0.1%) showed fluorescence. It was possible to detect a higher proportion (3 to 4%) of fluorescing cells in the preparations after transfection with the constructs pHL2017, pHL2146, and pHL2157.

To verify whether the truncated terminator also caused efficient termination in mouse cells, corresponding CAT constructs were produced with the murine rDNA promoter (pHL2170: 18 bp "Sal-box"; pHL2171: 12 bp "Sal-box"). B82 cells were used for the transfection and infection experiments. The CAT reactions performed 16 hours after infection had, with both

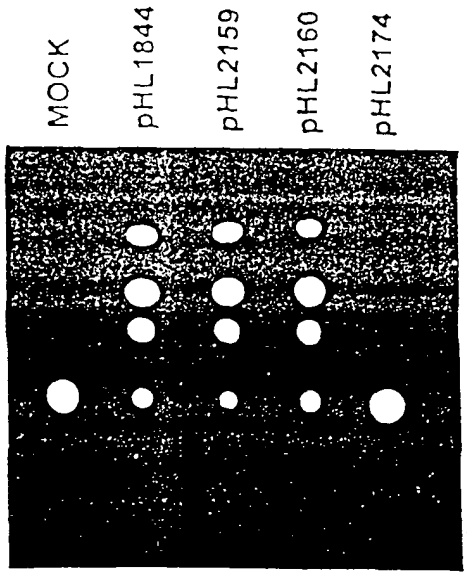


Fig. 3-49: CAT Reaction for the Investigation of the Efficiency of the RNA Polymerase I-Mediated Gene Expression after Truncation of the Murine rDNA Terminator Sequence
After transfection of COS-7 cells, the cells were processed 16 hours after infection and the CAT reaction was performed with the cell lysate (diluted 1 : 100).

constructs, a high signal strength (data not shown).

Accordingly, for the *in vivo* activity 223 bp of the human promoter region (or 254 bp of the murine rDNA promoter) and an at least 12-bp-long termination sequence are adequate for efficient initiation and termination of the RNA polymerase I transcription.

3.16.4 Optimization of the "PolI/PolIII Double Transcription System" by Truncation of the RNA Polymerase I Terminator Sequence

To produce a reporter plasmid with the double transcription system that contains the truncated RNA polymerase I terminator sequence, after cleavage of the plasmid pHX34 with *Nhe*I and *Pst*I, an *NheI/Pst*I fragment from pHL2159 was inserted into the vector fragment including 3564 bp (see Fig. 3-46). The resultant construct was named pHX45.

A comparative CAT analysis investigated how high the reporter gene activity is after primary and secondary infection in the double transcription system. For the transfection of 10^7 COS-7 cells, 5 μg plasmid DNA and 15 μl lipofectamine were used. The cells were processed 48 hours after the transfection (without infection). The construct pHL1993 (Fig. 3-44) showed, as anticipated, no CAT activity. The preparations after transfection with pHX33, pHX38, and pHX45 (Fig. 3-46) have a very high CAT signal strength (see Fig. 3-50a). A somewhat lower reporter gene expression is detectable with pHX34 and pHX39.

In a parallel preparation, the COS-7 cells were infected with FPV 24 hours after the DNA transfection. Processing occurred 24 hours after the infection (cf. Fig. 3-50b). With the exception of the double terminator plasmid pHX39, all constructs tested presented very high CAT activity.

The secondary infection of MDCK cells was performed with the supernatant of the primarily transfected and infected cells 24 hours after the primary infection. The cells were processed after 24 hours for the CAT analysis (see Fig. 3-50). The constructs pHX33, pHX38, and

pHX39 have no CAT activity. The preparations with pHL1993, pHX34, and pHX45 have a high CAT expression, which is at the same level with these constructs (see Fig. 3-50).

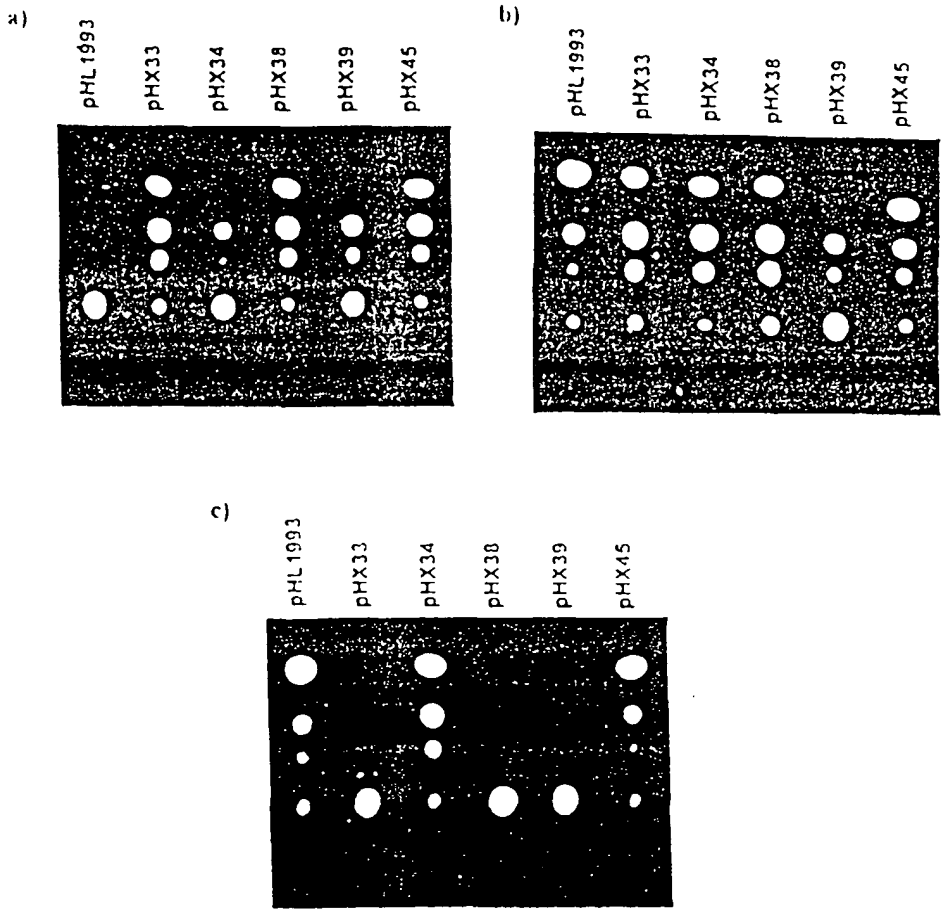


Fig. 3-50: CAT Reaction for the Analysis of the Optimized Double Transcription System
For the transfection of 10^7 COS-7 cells, 5 μg plasmid DNA/15 μl lipofectamine was used.
a) 48 hours after transfection (without infection), the cells were processed and the CAT reaction performed.
b) 24 hours after transfection, the cells were infected with FPV virus. 24 hours after the infection, the cells were processed for the CAT reaction.
c) 24 hours after infection of the COS-7 cells, an aliquot of the supernatant was applied to MDCK. 24 hours after the secondary infection, the cells were processed and the CAT reaction performed.

For comparison of the results from the CAT analyses, it was examined whether the expression of the green fluorescing protein can also be detected in the anti-parallel double transcription system. For this, an analogous series of constructs (pHX35, pHX36, pHX41, pHX42, and pHX47), presented in the table on the next page, was produced.

After the transfection of the GFP reporter plasmids (5 μg plasmid DNA/15 μl lipofectamine) in COS-7 cells, in each of two tissue culture dishes, one preparation, respectively, was also infected and the other not infected with FPV. The expression of the green fluorescing protein was tested by fluorescence microscopy and FACS analysis 36 hours after transfection (see Fig. 3-51).

In the noninfected cells, as anticipated, the constructs with a polymerase I transcription system pHL2017 and pHL2146 yielded no fluorescing cells. In the constructs which have the double terminator sequence including 173 bp (pHX35 and pHX41), it was possible in this manner to detect fluorescence in only a few cells (< 0.1%). A higher proportion of fluorescing cells (> 1%) was found in the variants without a terminator sequence (pHX36 and pHX42) or with a truncated terminator section (pHX47).

In the infected cells, in pHX41 only a few fluorescing cells were detectable (< 0.1%). All other constructs of this test series presented a significantly higher proportion of green fluorescing cells.

Table 3-1: Overview of the GFP and CAT R porter Constructs in the PolII//PolII Double Transcription System

The DNA elements that are not included in the respective constructs are marked with a ✓ (V: Vector; I: Inserted Fragment).

Plasmid (GFP)	Analogous to (CAT)	p_{HCMV}	a_{HBGH}	p_{Ih}	t_I	Cloning
pHX35	pHX34	✓	✓	✓	✓ 2 x 18 bp	V: pCMV1 *NheI/Bst*EII I: pHL2017 *NheI/Bst*EII
pHX36	pHX33	✓	✓			V: pCMV1 Δ*Bss*HII I: pHX35 Δ*Bst*HII
pHX41	pHX39	✓	✓		✓ 2 x 18 bp	pHX35 Δ*Bst*EII/*Stu*I (Klenow)
pHX42	pHX38	✓	✓	✓		pHX35 Δ*NheI/Asp*718 (Klenow)
pHX47	pHX45	✓	✓	✓	✓ 1 x 18 bp	V: pHX34 Δ*NheI/Pst*I I: pHL2146 Δ*NheI/Pst*I

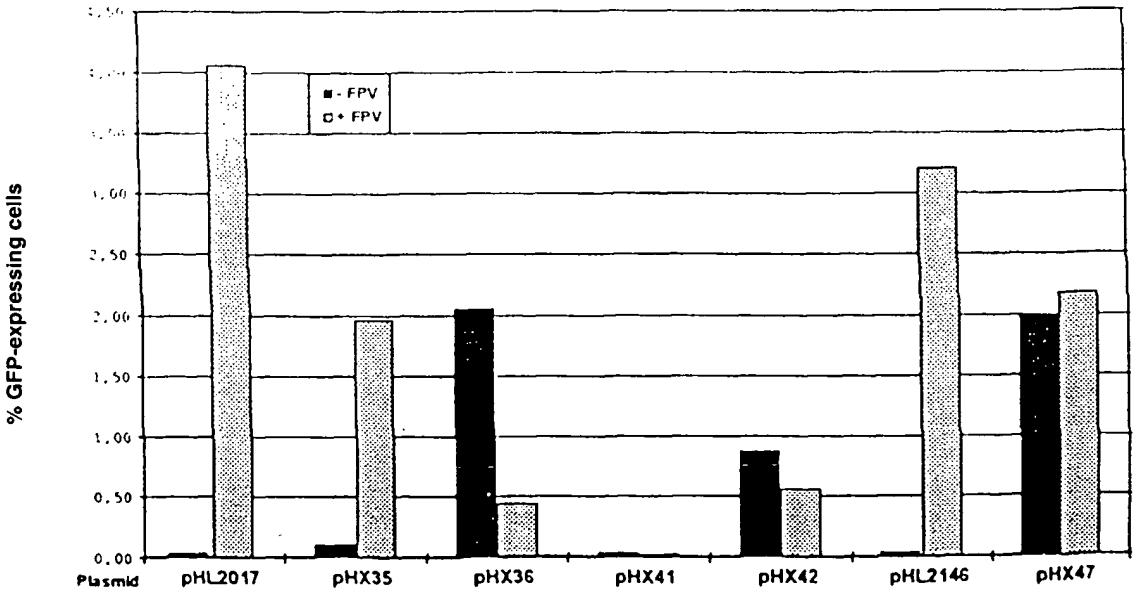


Fig. 3-51: FACS Analysis of the GFP-Expression in the Double Transcription System

After transfection of COS-7 cells with and without infection with virus, the proportion of fluorescing cells was determined by means of FACS analysis.

The structure of the plasmids pHX35, pHX36, pHX41, pHX42, pHX47 is presented in Table 3-1. pHL2017 and pHL2146 include only one RNA polymerase I transcription unit, whereby the terminator region of the latter plasmid includes only a single "Sal-box" (see Fig. 3-48).

The results of the CAT and the GFP reporter gene expressions demonstrate that the terminator sequence with two "Sal-box" sequences reduces the RNA polymerase II-mediated gene expression. In the CAT analysis, this is reflected in reduced CAT activity (pHX34 and pHX39). In the analogous GFP expression system (pHX35 and pHX41), the insertion of the terminator element even results in the fact that the level of expression of the reporter gene lies on the detection boundary. The substitution of the terminator sequence originally containing 173 bp by a 33-bp-long section (pHX47) with only one "Sal-box" results in a clearly detectable fluorescence in the GFP analysis. The CAT expression level after truncation of the terminator (pHX45) is also at the same level as comparative constructs without a terminator element. The CAT activities obtained as well as the fluorescence after passage of the daughter viruses on MDCK cells prove that the murine terminator sequence containing 33 bp in this system is adequate for the termination of the RNA polymerase I transcription in the monkey cells.

4 DISCUSSION

Influenza A viruses affect many different species in addition to humans. The varying pathogenicity of a virus strain for a specific host is caused by different factors. Besides ecological conditions, such as fish farming in China, its genetic characteristics are a decisive factor in the spread of a virus strain (Scholtissek & Naylor, 1988). The analysis of the molecular mechanisms of virus replication contributes to the understanding of the functions of the structures involved. Furthermore, the detailed knowledge of the regulation elements in the viral lifecycle enables selective construction of antiviral therapies and new improved vaccines.

Influenza A viruses have a segmented RNA genome with negative polarity. In order to be able to selectively alter the sequence of the viral RNA molecules, the RNA polymerase I transcription system was used within the framework of this research. After the introduction of the plasmid DNA into a eukaryotic cell and its accumulation in the cell nucleus, viral RNA is formed in the nucleolus by cellular RNA polymerase I. The reverse genetic system with the murine rDNA promoter and terminator established by Neuman *et al.* results in a higher transcription rate only in mouse cells (and with lower yields in closely related species). This property of the cellular RNA polymerase I complex is referred to as "species specificity" (Grummt *et al.*, 1986).

The replication capability of influenza viruses differs between various cell types and is a function of the virus strain used. After the transfection of the mouse fibroblasts cells, an FPV variant adapted to this cell type, i.e., an avian influenza virus was used without exception as a "helper virus" for the primary infection. Since it is known that human pathogenic viruses can replicate well on various primate cells (Govorkova *et al.*, 1996), it seemed reasonable to use

the human rDNA promoter, which was isolated for this and newly defined in its sequence, for the RNA polymerase I transcription in the cells.

After transfection of various primate cell lines with the CAT plasmid pHL1844, which includes a human RNA polymerase I promoter including 407 bp along with a murine terminator sequence, CAT activity was detected after infection with influenza helper virus. The constructs pHL1966 and pHL2168 with the reporter gene GFP also indicated, by the fluorescence of the reporter protein, the RNA polymerase I transcription of vRNA molecules. The constructs pHL1993 and pHL2017 with truncated promoter sequences (to position -223 referenced from the transcription start) reflected no attenuation in the reporter gene expression. Obviously, all significant *cis*-active signal elements that enable efficient transcription are included in the sequence elements present here. DNA sequences that enhance transcription, such as are frequently present in RNA polymerase II promoters at this interval are obviously not included in the region between 223 and 407.

The sequences of various mammalian rDNA promoters have only a few homologous sequences regions and are recognized by the associated transcription machinery only in closely related species (e.g., human and monkey) (Ling & Arnheim, 1994, Grummt *et al.*, 1982). Particularly noteworthy is a "Sal-box" (called T_0) approximately 180 bp upstream from the transcription start. Adjacent to this region is located the *cis*-regulatory DNA section referred to as UCE ("upstream control element"). The transcription factor UBF (upstream binding factor) binds to this sequence. UBF belongs to a family of DNA-binding proteins, whose DNA-binding domain is homologous with the non-sequence-specific DNA-binding domains of the HMG proteins ("high mobility group"). However, in contrast to the HMG proteins, the UBF protein has more than two such "HMG-boxes". On the carboxy-terminal end, this protein has an acidic domain that is essential for the transactivation (Jantzen *et al.*, 1992). Two isoforms of UBF are present in the eukaryotic cell, a 97-kDa protein (UBF1) and a protein truncated by 37 amino acids by differential RNA splicing (UBF2).

A second transcription factor, referred to in the human as SL1, in the mouse as TIFI-B, binds to UBF1 and results in a protein complex that can bind to the promoter sequence. This protein complex enables the formation of an initiation complex for the start of the transcription. Whereas UBF1, RNA polymerase I, and other associated factors are interchangeable between mouse and human, the transcription factor SL1/TIFI-B is the species-specific component of the pre-initiation complex. SL1/TIFI-B is a multiprotein complex consisting of the TATA-box binding protein (TBP) and three TBP-associated factors (TAFs) (Comai *et al.*, 1992, Eberhard *et al.*, 1993 in). In addition to TBP, the human complex consists of the transcription factors hTAF$_I$110, hTAF$_I$63, hTAF$_I$48; in the mouse the factors mTAF$_I$95, mTAF$_I$68, and mTAF$_I$48 have been identified. The TBP protein is also interchangeable between mouse and human so that for species-specific promoter recognition the TAF$_I$s are decisive (Rudloff *et al.*, 1994). TAF $_I$110 and TAF$_I$63 bind directly to the promoter sequence; in contrast, TAF$_I$48 interacts with UBF1 (Beckmann *et al.*, 1995). The cloning and sequencing of the factors involved revealed that the TAF$_I$s of mouse and human are from 66-80% identical. Compared to hTAF$_I$63, mTAF$_I$68 seems to have an additional zinc finger, which is possibly of significance for the species-specificity of the initiation complex (Heix *et al.*, 1997). In the human system, it was further possible to demonstrate *in vitro* that after purification of recombinantly produced TAFs and addition of UBF and a purified RNA polymerase I fraction, the initiation of the transcription is induced (Zomerdijk *et al.*, 1994). In an analogous experiment with appropriately produced mouse TAFs, it has however thus far not been possible to detect a productive initiation complex (Heix *et al.*, 1997).

The higher CAT activity in COS-7 cells compared to B82 cells is possibly attributable to the fact that, in addition to the technical advantages of this cell line, such as better transfection efficiency, the large SV40 T-antigen present in this cell type stimulates the RNA polymerase I transcription. As has been demonstrated by *in vitro* experiments as well as *in vivo* with SV40 infected cells, the activation of the transcription takes place through the direct interaction of the T antigen with the SL1 subunits TBP, TAF$_I$48, and TAF$_I$110 (Zhai *et al.*, 1997).

The detectable reporter gene activity of the plasmid constructs (pHL1844 and pHL1966), which include a chimeric RNA polymerase I transcription unit consisting of human rDNA promoter and murine terminator, demonstrated that the termination of the transcription also occurs efficiently in the primate cells. The mouse DNA elements of this section used here thus include all signal elements that are essential for the binding of the termination factors in the primate cells and precise formation of the 3' end of the primary transcript. For the efficiency of the transcription termination and precise formation of the 3' ends of the RNA, flanking sequences, in addition to the conserved "Sal-box" including 18 bp, are of significance (Kuhn *et al.*, 1988). After truncation of the terminator sequence to 33 bp (pHL2146/pHL2159) or 26 bp (pHL2150/pHL2160), a high reporter gene activity could nevertheless be detected. These constructs still contain in the terminator section one time (without repeat) the sequence element of the mouse (5'-AGGTCGACCAG-3') referred to as "Sal-box". The functionally analogous sequence in the human is identical on only 10 bp (5'-GGGTCGACCAG-3'). These results suggest that the human termination factor can bind on the murine terminator sequence. However, in the *in vivo* test performed here, it is necessary to consider that the quantity of precisely synthesized primary transcripts is measured only indirectly through the subsequent amplification with the influenza virus polymerase complex. Possibly, with the system used here (including the enhanced vRNA promoter) a small difference in the transcription rate for in the precision of the termination may not be detected. The constructs (pHL2147/pHL2174) with a Sal-box sequence including only 8 bp (5'-AGGTCGAC-3') present a clearly reduced reporter gene activity in the *in vivo* analysis. This result demonstrates that the efficient termination in the transfection system used here is also dependent on the presence of a complete "Sal-box" sequence. The deletion of three nucleotides evidently results in a poorer binding of the TTF-I termination factor on the target sequence. As a consequence, the RNA polymerase I protein complex cannot be stopped so that extended vRNA molecules develop on the 3' end, which then cannot be amplified by

the viral polymerase complex or can only be amplified much less well (with a template-internal start. [Sic: parentheses not closed]

The RNA polymerase I terminator elements include no palindromic sequences and function only in one orientation. Furthermore, the termination reaction is specific for the RNA polymerase I; thus, the murine termination factor terminates the transcription of the RNA polymerase I from yeast as well, whereas the native RNA polymerases II and III are not terminated (Kuhn *et al.*, 1990). The fact that, after binding on the target sequence, the termination factor not only acts as an obstacle to the RNA polymerase I, but interacts specifically with the RNA polymerase I complex follows in particular from the observed hydrolysis of the terminal nucleotide residue on the primary 3' end until the formation of the secondary 3' end of the RNA polymerase I transcripts. (Kuhn *et al.*, 1988; Zobel *et al.*, 1993).

The cloning and functional characterization of the cDNA, which encodes for the murine termination factor (mTTF-I), revealed a modular structure of this protein. By deletion analysis, three regions were classified: one N-terminal region not necessary for the termination reaction, a central domain, and a C-terminal DNA-binding domain, both of which are necessary for the transcription termination. The C-terminal region has homologies with the DNA-binding domains of the proto-oncogene c-Myb and the yeast transcription factor Reb1b. The DNA-binding domain of these proteins is characterized by a few conserved tryptophan residues. Substitution of the tryptophan residue at position 688 by a lysine residue results in a protein that can no longer bind on the target sequence. This result supports the hypothesis that mTTF-I, like c-Myb, has a DNA-binding domain that consists of multiple α-helices (Evers *et al.*, 1995; Saikumar *et al.*, 1990). The tryptophan residues form a hydrophobic backbone for the stabilization of the α-helix structure; adjacent basic and polar amino acids could then mediate the specific contact with the nucleotides of the "Sal-box".

The characterization of the cDNA of the human transcription termination factor (hTTF-I) revealed that this protein consists of 886 amino acids. The C-terminal 400 amino acids of the murine and human factors are 80% identical and 85% similar. The N-terminal

region is, however, different; it contains as an absolute characteristic many basic amino acids (Evers & Grummt, 1995). Despite the high homology in the DNA-binding domain, procaryotically expressed and purified mTTF-I protein does not bind to "human" Sal-box oligonucleotides, whereas the human factor recognizes both the homologous and the heterologous murine DNA sequence. *In vivo* experiments after transfection in mouse fibroblasts of chimeric constructs with a murine RNA polymerase I promoter and a human terminator sequence resulted in the termination reaction only after co-expression of hTTF-I (Evers & Grummt, 1995). These results show that, despite the high homologies in the DNA-binding domain of the proteins and just as with the associated "Sal-box" DNA elements, there are differences in the binding reactions.

Within the framework of this research, it turned out that both in COS-7 and B82 cells the termination of the transcription occurs after truncation of the murine 12 Sal-box to 12 bp. These results agree for the human situation with the results of the Grummt research group. It was demonstrated there that for the termination of the transcription 11 bp of the human terminator sequence (5'-GGGTCGACCAG-3') are adequate (Pfleiderer *et al.*, 1990). Since the murine Sal-box has the sequence (5'-AGGTCGACCAG-3') the factor from monkey cells also recognizes the murine sequence. The truncation of the murine Sal-box to 12 bp, which nevertheless results in a termination reaction in murine cells, is surprising, since *in vitro* transcription experiments after insertion of 7 bp between the two conserved elements AGGTCGACCAG and TCCG resulted in no detectable termination (Grummt *et al.*, 1986; Bartsch *et al.*, 1987). Since the element TCCG is no longer present in the *in vivo* transfection/infection experiments conducted here, the 12 bp are adequate for the binding of mTTF-I in mouse cell. To clarify that the human factor recognizes both the murine and the human sequence, but the murine factor only recognizes the native sequence, the different flanking sequences of the Sal-box must be responsible. This is again improbable since it had been demonstrated for the human sequence that 11 bp are adequate for the termination (Pfleiderer *et al.*, 1990). However, if we compare the analyses performed *in vivo* and *in vitro* more carefully, the sequence 5-AGGTCGACCAGT-3' was used for the murine Sal-box,

the sequence 5'-GGGTCGACCAGC-3' as the human Sal-box. Whether these flanking nucleotides are involved in the binding of the murine termination factor could be verified experimentally. A reporter gene construct with influenza-specific segment ends between a murine RNA polymerase I promoter and a human "Sal-box" sequence (or with the nucleotide substitutions to be differentiated) presents a positive result after transfection and infection if the target sequence is specifically recognized; no reporter gene activity or very reduced reporter gene activity if the sequence results in no bond or in a relatively weak bond.

Pleschka *et al.* (1996) also used the human rDNA promoter with a length including roughly 250 bp for the selective mutagenesis of influenza A viruses. In contrast with the system used here with a RNA polymerase I specific termination and processing sequences, in that case, the precise 3' end of the RNA primary transcript was formed by autocatalytic cleavage by a hepatitis delta ribosome sequence. For the primary infection, the human cell line 293 was used there.

In summary, it can be noted that for the *in vivo* expression in the plasmid-based RNA polymerase I transcription system after transient transfection of mammalian cells, the following sequence elements are adequate for a high transcription rate:

1. An rDNA promoter with a sequence including roughly 200 bp upstream from the transcription start including the so-called "T_0-Sal-box".
2. For the termination of the transcription, a single "T_1-Sal-box" including only 12 bp is adequate.
3. A C-rich sequence element of, for example, 15 bp between the 3' end of the sequence to be transcribed and the "Sal-box", which is important for the precise formation of the secondary 3' RNA end occurring after the stop of the RNA polymerase I (Zobel *et al.*, 1993).

For the use of the RNA polymerase I transcription system, it was first necessary to establish a cloning process that permitted site-specific insertion of cDNA between rDNA promoter and terminator. For this, restriction endonucleases of type IIs ("bracket enzymes") were used (see Fig. 3-6, Fig. 3-7). For both the murine and the human transcription system, plasmid factors with two recognition sequences of the restriction endonucleases *Bsm*BI were constructed (Fig. 3-8, Fig. 3-39). After amplification of PCR fragments that contain the desired 5' and 3' cDNA influenza sequences, these can then, after cleavage, be inserted directly into the vector. Since the overhanging ends are freely selectable in this process, the coupling of DNA fragments is possible without the introduction of foreign sequences. This site-specific fusion of two DNA molecules was a prerequisite for the fact that after the introduction of the recombinant plasmid molecules in the eukaryotic cells, RNA transcripts that have authentic influenza sequences on both their 5' end and 3' end were formed.

The genomic RNA of influenza A viruses consists of eight different RNA segments with negative polarity. These RNA segments are complexed both in the virions and in the infected cell with viral NP proteins and the three polymerase subunits. Each of these RNA molecules includes one or more open reading frames (ORF), which are flanked by short noncoding sequences on both ends. The noncoding sequences have the following characteristic structure: The first 12 and 13 nucleotides on the 3' and 5' end are highly conserved among all various RNA segments and virus strains. This section has partial complementarity, particularly the nucleotides 11, 12, 13 are complementary to the nucleotides $\overline{10}$, $\overline{11}$, $\overline{12}$. The following nucleotides 14, 15, and 16 on the 5' end of the vRNA of the same segment are likewise complementary to the nucleotides $\overline{13}$, $\overline{14}$, and $\overline{15}$ on the 3' end of the same segment. These nucleotide positions are, however, not conserved in comparison with the various RNA segments, but have the same sequence for a specific segment in all virus strains. A nucleotide sequence of from 5 to 7 uridine residues is found immediately following

the complementary region on the 5' end of the vRNA and is important for the polyadenylation of viral mRNA molecules during transcription (Li & Palese, 1994).

For the *in vivo* analysis, the entire encoding sequence of the respective influenza gene was precisely exchanged for the reporter gene CAT. The constructs investigated, in which the terminal, noncoding sequences of the segments 2, 4, 5, and 7 were present, demonstrated high reporter gene activity both in the primary and in the secondary infection (see Section 3.4). In other experiments, the encoding region of segment 8 (NS) was replaced nucleotidelike by the reporter gene CAT with the help of the RNP transfection system (Luytjes *et al.*, 1989). In this system as well, the noncoding sequence elements of this segment were adequate for detectable CAT activity. This means that in each case in these sections all signal elements are contained that are necessary for the replication and transcription of the viral RNA. The CAT expression after passage further means that these regions are also adequate in all segments investigated for the packaging of the (recombinant) vRNA molecules formed in new daughter virus particles.

The invariant 5' and 3' ends (13/$\overline{12}$ nucleotides) of the genomic RNA — interpreted as double-stranded ('panhandle') — have sequences largely complementary to each other, interrupted by two possible G:U-base pairings U3-G$\bar{3}$ or G5-U$\bar{5}$ and by a lack of pairing at position 8 (A8-C$\bar{8}$), as well as a non-paired 'angle nucleotide' A10 in the 5' region. However, the complementary cRNA end structure includes in this interpretation at position 3 and 5 two nucleotide non-pairings (C3-A$\bar{3}$, A5-C$\bar{5}$) and a U8-G$\bar{8}$ nucleotide pair as well as an non-paired U$\overline{10}$ on the 3' end. Although the two structures have a different sequence and structure, they are recognized by the viral polymerase complex and, consequently, used for the initiation of the RNA synthesis. Through nucleotide exchanges at positions$\bar{3}$, $\bar{5}$, and $\bar{8}$, the 3' vRNA promoter sequences was altered such that the terminal nucleotide can form, with the 5' end, a complete RNA double strand including nine nucleotides in each case, followed by six base pairs beyond the angle nucleotide A10. In the CAT analysis performed, this construct (pHL1169) presents greatly increased activity compared to the wildtype construct

(pHL1168) (see Fig. 3-5). These results agree with those of G. Neumann obtained with the plasmid constructs pHL926 and pHL1104 (Neumann & Hobom, 1995). An increased CAT activity due to the altered vRNA sequences on the 3' end also appeared in the human transcription system. After transfection of COS-7 cells and subsequent influenza infection, the construct pHL1844 with altered 3' vRNA ends yielded a significantly higher CAT expression rate than the comparative construct (pHL1863) with wildtype sequences (see Fig. 3-38).

Analogously to the vRNA molecules present after the primary transcription, the reporter gene CAT with flanking ends of segment 5 was also inserted in antigenomic orientation between rDMA promoter and terminator such that after transfection cRNA molecules should be synthesized by the cellular RNA polymerase I. The detectable CAT activity after transfection and infection shows that the cRNA promoter is recognized by the viral polymerase complex, a situation which results in the synthesis of vRNA (see Fig. 3-18). The vRNA is then used as a template for the synthesis of mRNA, which is ultimately translated into protein. Segment 5 (NP) has on the 5' end of the cRNA at position four a guanosine residue; segment 4 (HA) has at this position an adenosine residue. The construct pHL1399 thus has four nucleotide exchanges at the positions 3, 4, 5, and 8 in comparison with the wildtype construct pHL1398 on the 5' end of the cRNA. These exchanges (C3→U3, G4→A4, A5→G5, G8→A8) correspond on the vRNA level to the complementary nucleotides on the 3' end ($A\bar{3}$, $U\bar{4}$, $C\bar{5}$, $U\bar{8}$) as found in the "promoter-up" mutant pHL1104 (or pHL1169). In both situations, the refined complementary sequences of the RNA ends could result in the fact that after RNA polymerase I transcription RNA molecules with a double-stranded end structure are formed, which are then not broken down by cellular RNAs. An additional possibility consists in that the terminal double strand is better bound by the viral polymerase complex. Consequently, additional constructs that have no permanent terminal cRNA double-stranded structure were tested. The variant pHL1483 has on the 5' end in the cRNA position 5 an adenosine residue corresponding to the wildtype situation and a cytosine residue in position $\bar{5}$. This construct has high promoter efficiency. This result agrees with the mutant pHL1102 with HA-specific

ends (Neumann & Hobom, 1995). This construct results after transfection in vRNA primary transcripts with the nucleotides A$\overline{3}$ and U$\overline{8}$ on the 3' end and also has high CAT activity. These and further results demonstrate that the terminal promoter region is not only recognized as a double strand by the influenza polymerase complex, but that also a nucleotide-specific recognition occurs. For the high promoter activity, the nucleotides at positions 3 and 8 are particularly significant. Thus, the conversion from A8→G8 results in the loss of promoter efficiency (pHL1485). Likewise, in the analogous vRNA construct, which has at this position on the 3' end a U$\overline{8}$→C$\overline{8}$ substitution (pHL1102), no CAT activity was detected (Neumann & Hobom, 1995). The fact that, in addition to position 8, the nucleotide at position 3 is also nucleotide-specifically recognized is demonstrated by the mutant (pHL1484) with the exchange of U3→C3,which also permits no promoter performance.

The terminal structures of the wildtype sequence as well as the "promoter-up" variants are both recognized by the viral polymerase complex. Since these end regions exert an influence on the complex processes of replication and transcription, it seems unlikely that the overactive variants form a secondary structure completely different from the wildtype structure. The improved promoter efficiency is more likely attributable to smaller structural differences. Possibly, the bond between the nucleotides U3 and A8 at position 3 and 8 in the pHL1483 compared to the wildtype nucleotides C3 and G8 of the cRNA promoter and the amino·acid residues of the polymerase complex important for the bond is improved. For the bond, the subunits PB1 and PA are particularly important since both are necessary for replication (Nakagawa *et al.*, 1996).

Various models have been developed for the structure of the terminal sequences of the vRNA. According to the "panhandle" model, the terminal nucleotides are present — incompletely — base paired. This structure was proposed because in electronic microscopic investigations terminal double-stranded RNP structures had been observed in virions (Hsu *et al.*, 1987). Based on RNA polymerase binding analyses *in vitro* transcription reactions, Fodor *et al.* developed the "RNA-fork" model (Fodor *et al.*, 1994, Kim *et al.*, 1996). According to this idea, only the nucleotides (11-16 and $\overline{10}$ - $\overline{15}$) are present in base pairs whereas the proximal

nucleotides are supposed to be present as single strands. After *in vivo* analyses with complementary double substitutions, the "corkscrew" model was described by Flick *et al.* (1996) for the vRNA promoters. Here, the nucleotides 2:9 and 3:8, respectively, form two strand-internal base pairings on the 5' and on the 3' end. Via this conformation, the nucleotides then form, on positions 4 through 7 in each case, a single-stranded section of the structure, apparently with outwardly rotating base 5 or $\bar{6}$.

If one also assumes a "corkscrew" conformation for the cRNA promoter structure, this would yield a C3-G8 base pair for the wildtype construct (pHL1398); the improved promoter variants (pHL1399 and pHL1483) have at these positions a U3-A8 nucleotide pair. The variants with non-detectable promoter performance have no base pairing potential at the sites in question with C3-A8 (pHL1484) or U3-G8 (pHL1482, pHL1485). TheU3-G8 base pairing permits the formation of a double-stranded segment in the cRNA conformation; however, after copying in vRNA an A/C the nucleotide constellation is present. Accordingly, the analogous mutant on the vRNA level presents no CAT activity (pHL1103; Neumann & Hobom, 1995). The high promoter efficiency of pHL1399 and pHL1483 could presumably be caused by the fact that the "corkscrew" structure is retained with the two base pairings 2:9 and 3:8 and the interaction between the RNA and the amino acids important for the bonding is improved by the nucleotide substitution. In contrast to the constructs pHL1482 and pHL1485, the variants with high promoter efficiency could form A-U base pairings both in the vRNA and in the cRNA promoter structure. However, so that both the replication and/or transcription are not impaired, the dominant "corkscrew" structure that is important for the activity must be maintained both by the cRNA and the vRNA promoter. The greater promoter activity of the constructs with U3-A8 base pairs compared to the wildtype situation C 3-G 8 must then result from the fact that the nucleotides involved (U3-A8 in the cRNA or $A\bar{3}$ -$U\bar{8}$ in the vRNA) are better bound by the polymerase complex than the corresponding G-C nucleotide pairs.

The deletion of the "angle" nucleotide U$\overline{10}$ of the cRNA promoter resulted in the loss of the promoter activity (pHL1514). After insertion of an adenosine residue at the 3' end (pHL1513) as well as by flipping the angle (pHL1450), no CAT activity can be detected (see Fig. 3-23). These results show that the angle position represents an important structural element that forms a flexible joint between the proximal and distal element of the cRNA promoter.

The mutation analysis with complementary double substitutions under this total cRNA promoter region at position $\overline{11}$ on the 3' end and position 12 on the 5' end showed high promoter activity (see Fig. 3-25) for the constructs with Watson-Crick base pairing (G11-C$\overline{12}$, C11-G$\overline{12}$ and A11-U$\overline{12}$, U11-A$\overline{12}$). No promoter activity was measurable when the nucleotides opposite each other could form no base pairing at the relevant positions (e.g., G11-G$\overline{12}$, G11-A$\overline{12}$). Also, in a mutation analysis performed by Pritlove *et al.*, the constructs with nucleotide exchanges C$\overline{12}$ →G$\overline{12}$ and C$\overline{12}$ →A$\overline{12}$ at the 3' cRNA end had no promoter activity in an *in vitro* transcription reaction (Pritlove *et al.*, 1995). The substitution C$\overline{12}$ →U$\overline{12}$ resulted in this analysis in a very slight but detectable activity of the promoter structure. In the CAT analyses performed here, reporter gene activity could be detected neither in the primary nor in the secondary infection. These slight differences are possibly attributable to the different detection systems. For the reaction, synthetically produced RNA templates and purified polymerase complex were used. In contrast, in the analyses performed here, complete cRNA molecules with the corresponding ends were formed in the cell and subsequently recognized by the viral polymerase complex, and copied in vRNA. Subsequently the vRNA is used as a template for the synthesis of mRNA.

In the CAT analysis as well, the construct pHL1358 with A12/C$\overline{11}$, with which vRNA is synthesized after primary transcription, shows no detectable CAT activity (Flick *et al.*, 1996) in agreement with the nondetectable promoter activity of the cRNA construct U11-G$\overline{12}$ (pHL1647). The A-U base pairing (pHL1655 U11-A$\overline{12}$) as well results, as with the vRNA construct (pHL1386A 12-U$\overline{11}$), in detectable promoter activity. The results demonstrate that

for the binding of the viral polymerase complex of the distal region of the vRNA promoter as well as the cRNA section complementary thereto must be present as an RNA double strand. The nucleotides in question are not specifically recognized. This could argue for an interaction of the polymerase proteins with the phosphate backbone of an RNA helix in this region.

For the investigation as to whether, in addition to the highly conserved terminal nucleotides, which are identical in all eight segments, the segment-specific nucleotides are also significant for the viral replication cycle or even for a segment-specific recognition, combinations of different segment and structures were first produced. The structure variants resulted in variably high CAT activities (see Section 3.5). Accordingly, the relevant sequence sections have a critical significance for the viral mRNA expression rate. The nucleotides 3-16 at the 5' end and $\overline{12}$-$\overline{15}$ at the 3' end of the vRNA could form nucleotide pairs in segment 4 GUGU-CCUA, in segment 5 GUA-CAU, in segment 2 GU-CA, in 7 UAG-AUC. After combination of these 5' and 3' sequence elements, all those variants (pHl1318 GUAU-CCUA, pHL1298 GUG-CAU, pHL1425 UAG-GUU) that then contain even more base pairings present high promoter efficiency. In these mutants, the nucleotides 11-16 on the 5' end and opposite the nucleotides $\overline{10}$-$\overline{15}$ on the 3' end can form an RNA double strand of six (or five) base pairs. An RNA double strand, adjacent to the oligo-U sequence represents a regulation element for the polyadenylation of the mRNA (Li & Palese, 1994). The constructs without complementary nucleotide regions (pHL1302, pHL1321, pHL1297, pHL1421, pHL1301, pHL1319, and pHL1423) have either reduced promoter activity or none. However, a few variants that can form a lengthened double strain only by rotating out a base (pHL1302, pHL1321) have high promoter activity. The structural elements involved then apparently inhibit the mRNA synthesis only to a slight extent. Additional mutation analyses would be necessary for a more precise characterization of the extent that the distance of this double-stranded region from the angle position of the vRNA promoter is variable.

The nucleotides 16 through 45 of the noncoding region of segment 5 are highly conserved. Among the various influenza strains, there are only a few nucleotide exchanges in this section. Particularly noteworthy is a sequence region from position 20 through 36 with a palindromic sequence, which could form a "stem loop structure". However, the deletion of this region (pHL1595) presents no weakening of the CAT reaction (see Fig. 3-27). If the flanking sequence elements (nucleotides 16-20 and 21-35) are removed, this likewise results in a high reporter gene activity (pHL1596). And even the total deletion of the complete subterminal sequence elements does not lead to a decrease in the CAT activity (pHL1660). These sequence sections are thus not essential for the replication and transcription of the recombinant RNA segments. As the detectable mRNA synthesis after passage on MDCK cells proves, the still present 16 nucleotides on the 3' end of the vRNA and 23 nucleotides on the 5' end are also adequate for the packaging of the vRNA molecules in daughter virus particles.

Using the RNP transfection system, Zheng *et al.* (1996) performed mutation analyses on the noncoding region of NA (segment 6). Here, deletions between position 15 and the start codon had no effect on the quantity of viral RNA molecules in virions and in infected cells. Deletions of the sequence between the stop codon and the oligo-U sequence also resulted in a high NA-RNA concentration. The combination of the deletion mutants on the 5' and on the 3' ends resulted, however, in a reduction of the synthesis of the corresponding recombinant RNA molecules. That last result is noteworthy since in the double mutant only the nucleotides sequence 5'-UUAA-3' on the 3' end of the vRNA is deleted in comparison with the 5'-deletion mutant. The quantity of NA-RNA is then reduced with this construct 60-fold in virions and infected cells. The authors conclude from these results that the nonconserved region of NA plays an important role in the replication of this segment. In contrast, the results obtained for segment 5 within the framework of this research argue that this sequence region is not significant for the replication of this segment. In light of the expression performance of the promoter variants selected as a starting point, it is not possible to conclude that these segments are possibly important for the fine regulation of mRNA

synthesis or stability, discernible only in conjunction with the weaker wildtype promoter.

Enami *et al.* (1994) demonstrated using the RNP transfection system, that NS1 stimulates the translation of NP-CAT mRNA. The nucleotide sequence $G_{12}G_{13}U_{14}A_{15}G_{16}A_{17}U_{18}A_{19}$ of the cRNA was identified there as a *cis*-active sequence. Park & Katze (1995) demonstrated that the nucleotides 1-21 are significant for the regulation of the translation. These results agree with the results found here to the extent that the "stem loop" structure (position 21-38) is not a decisive factor for the initiation of translation, since no effect due to deletion of the mentioned sequences could be established.

Since the recombinant segment with the "minimal" noncoding region of segment 5 is also present in addition to the eight essential viral vRNA segments in the daughter viruses, all signal elements necessary and sufficient for the packaging of the RNA molecules are present in this subregion. After synthesis of the vRNA, the binding of the polymerase complex on this terminal region as well as the addition of NP molecules on the vRNA occur. The RNP particles formed are transported to the cell membrane and packaged during virus maturation in the daughter virus particles. In this case, the packaging of the eight RNP particles and of the additional ninth segments occurs in a static process. Consequently, an independent packaging signal distinct from the promoter structure does not exist; it could not be found in any of virtually 300 mutants of the viral promoter structure (R. Flick, personal report).

For the improvement of the RNA polymerase I transcription system, the reporter gene GFP was inserted, flanked by influenza-specific ends, between the human rDNA promoter and the murine terminator. After transfection and infection, the expression of the reporter gene was detectable by the fluorescence of the cells. For the detection of the expression, it was, however, necessary to use a GFP mutant that has a threonine residue at position 65 instead of a serine residue. This substitution causes a dramatic change in the spectroscopic characteristics: The wildtype molecule has two excitation maxima at 396 nm and 475 nm; in

contrast, the "S65T" mutant has only a single absorption maxima at 489 nm. This "S65T" mutant has, however, a roughly six-fold higher fluorescence emission compared to the wildtype molecule (Heim *et al.*, 1995).

The three-dimensional structure of the wildtype and of the "S65T" mutant were determined independently of each other (Ormö *et al.*, 1996, Yang *et al.*, 1996). The molecule consists externally of a 12-strand β-barrel, which forms a cylindrical outer shell. A single α-helix passes through the center of this cylinder. The fluorophoric group cuts the α-helix structure in the center and is thus shielded from the surrounding solvent. This structure is embedded in an unusually hydrophilic area of the nuclear region. The differences in the three-dimensional structure between the wildtype and "S65T" are located in the surroundings of the chromophore. All rearrangements are caused by the presence or absence of a methyl group of the amino acid residue at position 65. In the wildtype structure, the hydroxyl group of Ser-65 is held at a different position from the corresponding oxygen in the S65T mutant. The conformation changes caused by this resulted in different hydrogen bonds of the adjacent amino acids (e.g., Glu222, His148). Consequently, in the wildtype structure, the chromophore is present largely in an electrically neutral state, which is the cause of the shortwave absorption band. The longwave absorption band appears in the S65T mutant as a single band, since, because of the amino acid rearrangement, the chromophore has a negative charge due to deprotonation of Tyr66 (Brejc *et al.*, 1997).

This substitution Tyr66→His changes the spectroscopic properties such that after excitation with light of a wavelength of 382 nm, the fluorescing emission takes place at 448 nm (Heim *et al.*, 1994). This means that, after excitation, this variant no longer "shines" green, but blue. The use of this GFP mutant in the influenza system (or of a variant with additional amino acid exchanges, Heim & Tsien, 1996) should permit the simultaneous detection of a "green" and "blue" fluorescing protein.

The additional substitution of Phe64 to Leu64 presents no improved fluorescence characteristics in the influenza system (Cormack *et al.*, 1996).

In addition to changing the fluorescence characteristics through amino acid substitutions in the fluorophoric group, GFP mutants have been described that contribute to better folding of the protein at 37°C (Val163→Ala; Ile167→Thr; Ser175→Gly; Siemering *et al.*, 1996). To increase the translation rate of the GFP protein, the sequence originally present in *Aquorea victoria* was replaced by the total synthesis of the gene with the use of the codon triplets preferred in human genes (in third position C or G; Zolotukhin *et al.*, 1996). The use of this variant in the influenza system should, consequently, also be capable of resulting in an

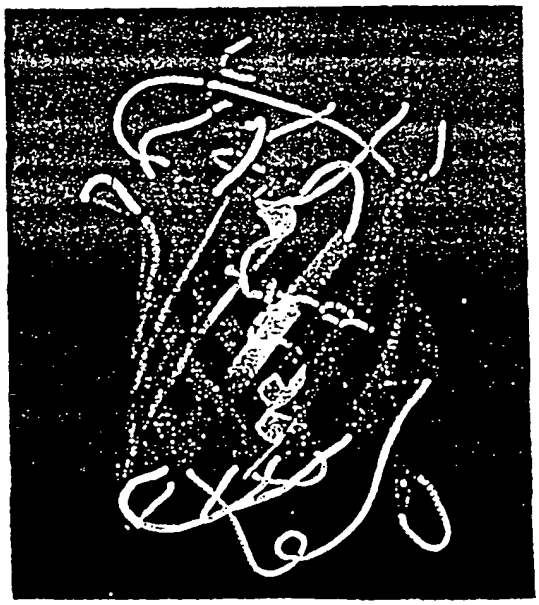


Fig. 4-1: Th Three-Dim nsi nal Structure of the Gr en Fluorescing Protein
In the center of the molecule, there is an α-helix that is surrounded by a cylindrical, 11-stranded β-pleated-sheet structure (green). The chromophoric group is located in the center of the molecule and is thus completely shielded from the surrounding solvent (Illustration from Brejc *et al.*, 1997).

increased fluorescence yield.

The direct *in vivo* detection of the reporter gene GFP facilitates the control of test conditions, such as the determination of transfection efficiency. In addition to this general improvement of the influenza vector system, it is possible, through the use of the green fluorescing protein and even of GFP fusion constructs through an indirect selection process, to increase the proportion of recombinant viruses in the total population. After primary or secondary infection, infected and simultaneously fluorescing cells can be separated by fluorescence activated cell sorting from infected but nonfluorescing cells. After additional incubation of the cells, after this preselection, the proportion of recombinant viruses in the supernatant will be increased compared to nonselection.

Because of compact structure of the GFP molecule, the fluorescing behavior as part of a fusion protein should be kept substantially unaltered. Since already after the first passage, the proportion of GFP expressing cells was roughly 50% (see Fig. 3-43), the influenza expression system can also be used for the eukaryotic protein expression with subsequent protein purification. Through the fusion of the GFP molecule with a protein of interest, direct detection is possible and thus the level of expression directly visible. Compared to the RNA polymerase II-mediated protein expression after transient transfection, the influenza vector system has the advantage that after transfection of the RNA polymerase I plasma constructs with subsequent infection, recombinant daughter viruses are formed. These virus particles contained in the supernatant are then passaged on fresh cells. In the infection, more cells receive the genetic information than after a DNA transfection. The replication of the genetic information through recombinant viruses in the passage, for which only the use of a buffer (PBS +) is necessary, also brings a cost advantage compared to a more expensive lipofectamine DNA transfection.

The vector system developed within the framework of this research provides the prerequisites to simplify or to finally enable the use of influenza vectors for the channeling of genes into eukaryotic cells. Thus, with cell types, in which low expression rates of a foreign gene, or

none at all, can be obtained by other processes, such as liposome-DNA transfection or retroviral infection (Verma & Somia, 1997), the expression of the fluorescing protein can, for the first time, be detected *in vivo* after infection with recombinant "GFP influenza viruses". If the expression of the reporter gene GFP is to be detectable, the plasma vector system developed here can be used to insert the cDNA of interest into the RNA polymerase I vector system (see Fig. 3-32 through Fig. 3-35). After production of recombinant influenza viruses, the level of expression of the foreign gene can be determined immediately after infection of the new target cells. Consequently, this gene transfer system based on influenza A viruses represents a further possibility of introducing genes in an efficient manner into eukaryotic cells and is, consequently, an interesting alternative to other viral vector systems (such as retroviruses or adenoviruses).

5 ABSTRACT

The transient RNA polymerase I expression of influenza vRNA or even cRNA with subsequent amplification by the viral RNA polymerase complex enables the selective mutation analysis of influenza A viruses.

This DNA transfection system was used to investigate the function of the 5' and 3' terminal nontranslated sequences of segment 2, 4, 5, and 7. The successful expression, amplification, and packaging of gene constructs in which the entire coding sequences of the respective influenza gene is precisely exchanged for the reporter gene (CAT) makes it clear that all necessary signal elements are localized in these terminal sections. Contrary to previous results, which, based on *in vitro* experiments, primarily characterized only the 3' promoter section as adequate for the binding of the viral polymerase complex, the *in vivo* experiments conducted here show that both the 5' ends and the 3' ends of the terminal subregions are necessary for viral replication transcription.

Through various deletion mutants in the nontranslated segment-specific viral RNA segments, particularly at the 5' end of the cRNA of segment 5, it was possible to limit this signal region to 23 nucleotides on the 5' end and 16 nucleotides on the 3' end. Even the insertions of longer foreign sequences (more than 100 nucleotides) between the segment ends and the start of the reading frame only negatively affect the level of CAT activity to a slight extent.

Based on these mutation analyses, two series of plasmid vectors with rDNA promoter and terminator were constructed. In one group a multiple cloning sequence is located in different orientation between the terminal sequences of segment 5 with an optimized promoter region. A second group uses the "bracket enzyme" *Bsm*BI for a foreign-sequence-free insertion of influenza variants. For research in human and in primate cells as well as to increase the

efficiency of the transfection system, the murine rDNA promoter was replaced by the human rDNA promoter. The fluorescence of the green fluorescing protein (GFP) *in vivo* without the addition of a substrate enables the development of indirect selection methods using microscopy and FACS. In cotransfection processes or through GFP fusion constructs, an increase in the efficiency of this step is also possible through detection of this reporter protein.

The improvement of the RNA polymerase role in one transfection system thus enables the expression of a large number of homologous or heterologous gene segments. The resultant recombinant viruses can be investigated immediately for their immunogenic properties, a situation which promises to facilitate the development and production of attenuated live vaccines.

7 ABBREVIATIONS

°C	degree Celsius
µg	microgram
µM	micromolar
µmol	micromole
A	adenosine, ampere
AA	acrylamide
AA-bis	N, N-methylene-bisacrylamide
Ac	acetate
Amp	ampicillin
AMPS	ammonium persulfate
AS	amino acids [English AA]
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
CAM	chloramphenicol
CAT	chloramphenicol acetyltransferase
cRNA	complementary RNA (positive-strand RNA)
cDNA	complementary DNA
CoA	coenzyme A
CPE	cytopathic effect
cpm	counts per minute
d	deoxy
dd	dideoxy
DEPC	diethyl pyrocarbonate
dest.	distilled [English dist.]
DMEM	Dulbecco minimal essential medium
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
DOPE	dioleoyl phosphatidylethanolamine
DOSPA	2,3 dioleyoxy-N-[2(spermine carboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate
DTT	dithiothreitol
E. coli	*Escherichia coli*

EB	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
FKS	fetal calf serum [English FCS]
g	gram
G	guanosine
Gly	glycine
GTP	guanosine triphosphate
h	hour
His	histidine
kb	kilobase pair
l	liter
LB	Luria-Bertani medium
Leu	leucin
M	molar
m.o.i.	multiplicity of infection
mA	milliampere
min	minutes
ml	milliliter
mM	millimolar
MOPS	N-morpholino propanesulfonic acid
mRNA	messenger RNA
ng	nanogram
nm	nanometer
nmol	nanomole
NP	nucleoprotein
nt	nucleotide
NTP	nucleotide triphosphate
OD	optical density
pA	polyadenylation signal
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfu/PBE	plaque forming unit [PBE = German abbreviation]
pH	pontus hydrogenii (negative decadic logarithm of H_3O^+-ion concentration)
Phe	phenylalanine
pIh	RNA-polymerase I promoter (human)
pIICMV	RNA-polymerase II promoter (cytomegalovirus)
pIm	RNA-polymerase I promoter (murine)
pmol	picomole

PolI	RNA polymerase I
PolII	RNA polymerase II
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
SDS	sodium dodecylsulfate
Ser	serine
T	thymidine
TAE	Tris-acetate/EDTA-buffer
TBE	Tris-borate/EDTA-buffer
TdT	terminal deoxynucleotidyl transferase
TE	Tris-EDTA-buffer
TEMED	N, N, N', N'-tetramethylenediamine
Thr	threonine
tI	terminator of the RNA polymerase I
Tris	trihydroxy methylaminomethane
Tyr	tyrosine
U	unit (unit of enzyme activity)
U	uridine
UV	ultraviolet
V	Volt
v/v	volume percent
vRNA	viral RNA ((-) strand RNA)
W	Watt
w/v	weight per volume
w/w	weight percent
WT	Wild type

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Declaration

I hereby declare in lieu of an oath that I independently executed the present work and used only the aids indicated. I have indicated the source of all passages which were taken from other works in words or in meaning.

I have not submitted this work or parts thereof to any other examination committee for expert assessment.

Erich Hoffmann